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Biotechnology — Requirements for evaluating the performance of quantification methods for nucleic acid target sequences — qPCR and dPCR

Biotechnologie — Exigences relatives à l'évaluation de la performance des méthodes de quantification des séquences d'acides nucléiques cibles — qPCR et dPCR

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 276, *Biotechnology*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

This document has been developed to specifically support the analytical requirements with respect to quantification of specific nucleic acid sequences (targets). It can also benefit the broader biomanufacturing, bioscience research and development, industrial biotechnology, engineering biology and advanced therapeutics industries which need to demonstrate product quality based on measurement and quantification of specific nucleic acid targets.

Quantification of nucleic acid target sequences is a cross-cutting fundamental measurement that broadly impacts many aspects of biotechnology. For example, quantification of nucleic acid biomarkers for monitoring bioprocess efficiency and conformity with quality by design parameters for biopharmaceutical manufacture and industrial biotechnology, characterization of purity and quality of cell-derived advanced therapy medicinal products (ATMPs); assessment of gene copy number for evaluating the potency and efficacy of gene-based therapies and process control assays for gene editing and engineering biology applications.

The underpinning technique of polymerase chain reaction (PCR) has transformed the field of nucleic acid analysis, due to its robustness and simplicity. Technological advances in instrumentation have resulted in a wide range of PCR-based nucleic acid quantification approaches/instruments with subsequent developments such as:

— quantitative real-time PCR (qPCR) which offers methods for quantification of DNA and RNA molecules relative to a calibration material or independent sample, and

— digital PCR (dPCR) which offers the ability to perform SI traceable quantification through the concept of molecular enumeration without the need for a calibration curve.

However, performing nucleic acid quantification assays to a high standard of analytical quality can be challenging. For example, it is well known that impure or degraded nucleic acid extracts can affect the accuracy of quantification. Similarly, a poorly designed qPCR or dPCR assay with poor amplification efficiency and primer specificity will have an impact on accuracy of quantification. In addition, aspects such as calibrators, standard curves, data normalization and processing can have a large influence on the accuracy of quantitative measurement of nucleic acid targets.

This document is expected to improve confidence in the data produced, support selection and optimization procedures and provide supporting performance parameters that may be utilized during performance qualification of a particular measurement procedure for quantification of nucleic acid target sequences. Biotechnology and bioscience industry data with higher measurement confidence will enable data interoperability, improved product quality, reduced risks and costs and facilitate international trade.

In this document, the following verbal forms are used:

— “shall” indicates a requirement;

— “should” indicates a recommendation;

— “may” indicates a permission;

— “can” indicates a possibility or a capability.

Further details can be found in the ISO/IEC Directives, Part 2.

Biotechnology — Requirements for evaluating the performance of quantification methods for nucleic acid target sequences — qPCR and dPCR

1 Scope

This document provides generic requirements for evaluating the performance and ensuring the quality of methods used for the quantification of specific nucleic acid sequences (targets).

This document is applicable to the quantification of DNA (deoxyribonucleic acid) and RNA (ribonucleic acid) target sequences using either digital (dPCR) or quantitative real-time PCR (qPCR) amplification technologies. It applies to target sequences present in nucleic acid molecules including double-stranded DNA (dsDNA) such as genomic DNA (gDNA) and plasmid DNA, single stranded DNA (ssDNA), complementary DNA (cDNA), and single stranded RNA (ssRNA) including ribosomal RNA (rRNA), messenger RNA (mRNA), and long and short non-coding RNA [microRNAs (miRNAs) and short interfering RNAs (siRNAs)], as well as double-stranded RNA (dsRNA).

This document applies to nucleic acids derived from biological sources such as viruses, prokaryotic and eukaryotic cells, cell-free biological fluids (e.g. plasma or cell media) or in vitro sources [e.g. oligonucleotides, synthetic gene constructs and in vitro transcribed (IVT) RNA].

This document is not applicable to quantification of very short DNA oligonucleotides (<50 bases).

This document covers:

— analytical design including quantification strategies (nucleic acid copy number quantification using a calibration curve as in qPCR or through molecular counting as in dPCR, quantification relative to an independent sample and ratio measurements) and use of controls;

— quantification of total nucleic acid mass concentration and quality control of a nucleic acid sample including assessment of nucleic acid quality (purity and integrity);

— PCR assay design, optimization, in silico and in vitro specificity testing;

— data quality control and analysis including acceptance criteria, threshold setting and normalization;

— method validation (precision, linearity, limit of quantification, limit of detection, trueness and robustness) with specific requirements for qPCR and dPCR;

— approaches to establishing metrological traceability and estimating measurement uncertainty.

This document does not provide requirements or acceptance criteria for the sampling of biological materials or processing of biological samples (i.e. collection, preservation, transportation, storage, treatment and nucleic acid extraction). Nor does it provide requirements and acceptance criteria for specific applications (e.g. food or clinical applications where specific matrix issues can arise).

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO/IEC Guide 98-3:2008, *Uncertainty of measurement — Part 3: Guide to the expression of uncertainty in measurement (GUM:1995)*

ISO/IEC Guide 99, *International vocabulary of metrology — Basic and general concepts and associated terms (VIM)*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in ISO/IEC Guide 99 and the following apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
amplicon
specific DNA fragment produced by a DNA-amplification technology, such as the polymerase chain reaction (PCR)

[SOURCE: ISO 13495:2013, 3.3.1]

3.2
amplification plot
graph representing the generation of a reporter (usually fluorescent) signal during a qPCR or dPCR reaction

Note 1 to entry: For qPCR and some dPCR systems, the amplification plot shows the relationship between cycle number (x-axis) and fluorescence signal (y-axis).

Note 2 to entry: For end point dPCR, the fluorescent signal of each dPCR partition is displayed. For a single fluorophore, a one-dimensional amplification plot shows partition number (x-axis) against end point fluorescent signal (y-axis). A multi-dimensional amplification plot shows fluorescent signal for each detector channel on each axis.

3.3
calibration curve
standard curve
expression of the relation between indication and corresponding measured quantity value

[SOURCE: ISO/IEC Guide 99:2007, 4.31, modified — The notes have been deleted.]

3.4
calibrator
measurement standard used in calibration

Note 1 to entry: The term "calibrator" is only used in certain fields.

EXAMPLE A qPCR interplate calibrator sample is often included on each qPCR plate in a study comprising multiple qPCR plates or experiments to compensate for variations across plates due to instrument measurement factors such as baseline and threshold setting. The interplate calibrator contains the target sequence(s) detected by the PCR assay and is measured with the same PCR assays as the studied samples.

[SOURCE: ISO/IEC Guide 99:2007, 5.12, modified — The example has been added.]

3.5
cDNA
complementary DNA
single-stranded DNA, complementary to a given RNA and synthesised in the presence of reverse transcriptase to serve as a template for DNA amplification

3.6
copy number
number of molecules (copies) containing a specific nucleic acid sequence

[SOURCE: ISO 16577:2016, 3.28, modified — "of a DNA sequence" replaced with "containing a specific nucleic acid sequence"]

3.7
copy number concentration
number of molecules (copies) containing a specific nucleic acid sequence in a defined volume

3.8
quantification cycle
C_q
<qPCR> cycle at which the fluorescence from the reaction crosses a specified threshold level at which the signal can be distinguished from background levels

Note 1 to entry: Quantification cycle is a generic term which includes cycle threshold (C_t), crossing point (C_p), take off point and all other instrument specific terms referring to the fractional cycle used to quantify the concentration of target in the qPCR assay.

Note 2 to entry: The quantification cycle is based either on a threshold applied to all samples or on a regression analysis of the signal, for each sample.

[SOURCE: ISO 16577:2016, 3,32 modified according to MIQE Guidelines[1] — Notes 1 and 2 to entry have been added.]

3.9
delta C_q
ΔC_q
difference between two C_q values, $\Delta C_q = C_{q(1)} - C_{q(2)}$

3.10
digital PCR
dPCR
procedure in which nucleic acid templates are distributed across multiple partitions of nominally equivalent volume, such that some partitions contain template and others do not, followed by PCR amplification of target sequences and detection of specific PCR products, providing a count of the number of partitions with a positive and negative signal for the target template

Note 1 to entry: Nucleic acid target sequences are assumed to be randomly and independently distributed across the partitions during the partitioning process.

Note 2 to entry: The count of positive and negative partitions is normally based on end point detection of PCR products following thermal cycling, however real-time qPCR monitoring of PCR product accumulation is additionally possible for some dPCR platforms.

3.11
gene of interest
GOI
gene target sequence under investigation

3.12
lambda value
λ
mean number of targets per dPCR partition based on the fraction of droplets where amplification has occurred

Note 1 to entry: Essential dPCR quantities for calculation of lambda are the number of positive partitions (N_P) and the total number of partitions (N_T).

3.13
limit of quantification
LOQ
lowest concentration or quantity of the nucleic acid target sequence per defined volume that can be measured with reasonable statistical certainty consistently under the experimental conditions specified in the method

Note 1 to entry: Generally expressed in terms of the signal or measurement (true) value that will produce estimates having a specified coefficient of variation (CV).

[SOURCE: ISO 16577:2016, 3.91, modified — replaced "content of the analyte of interest" with "quantity of the nucleic acid target sequence", "amount of matrix" with "volume" and "relative standard deviation (RSD)" with 'coefficient of variation (CV)".]

3.14
limit of detection
LOD
measured quantity value, obtained by a given measurement procedure, for which the probability of falsely claiming the absence of a component in a material is β, given a probability α of falsely claiming its presence

[SOURCE: ISO/IEC Guide 99:2007, 4.18 definition for 'detection limit'; Notes to entry not included.]

3.15
linearity
ability of a method of analysis, within a certain range, to provide an instrumental response or results proportional to the quantity of nucleic acid target sequence to be determined in the laboratory sample

Note 1 to entry: In the case of qPCR, the cycle threshold is proportional to the logarithm to the base 10 of the quantity of nucleic acid target sequence.

Note 2 to entry: The term linearity is frequently linked with the linear range of the method and refers to the ability of a method to give a response or result that is directly proportional to the concentration of the nucleic acid target sequence.

[SOURCE: ISO 16577:2016, 3.92 modified — Notes 1 and 2 to entry added; "quantity of analyte" replaced with "quantity of the nucleic acid target sequence".]

3.16
measurand
quantity intended to be measured

Note 1 to entry: The specification of a measurand requires knowledge of the kind of quantity, description of the state of the phenomenon, body, or substance carrying the quantity, including any relevant component, and the chemical entities involved.

Note 2 to entry: In the second edition of the VIM and in IEC 60050-300:2001, the measurand is defined as the "particular quantity subject to measurement".

Note 3 to entry: The measurement, including the measuring system and the conditions under which the measurement is carried out, might change the phenomenon, body, or substance such that the quantity being measured differs from the measurand as defined. In this case, adequate correction is necessary.

EXAMPLE 1 Quantity of a gene target measured by PCR is influenced by the amplicon size of the PCR assay and fragment size of the template (~ < amplicon size).

EXAMPLE 2 Denaturation of DNA in a sample into ssDNA influences quantification by dPCR as the two strands are partitioned separately.

[SOURCE: ISO/IEC Guide 99:2007, 2.23, modified —Note 3 and examples have been modified, and Note 4 has been omitted.]

3.17
melting curve
analysis describing the dissociation characteristics of double-stranded DNA observed during heating

[SOURCE: ISO 16577:2016, 3.107, modified — Note 1 to entry has been deleted.]

3.18
melting temperature
T_m
temperature at which 50 % of double-stranded DNA helices are dissociated since a DNA helix melts in a temperature range rather than at one very specific temperature

[SOURCE: ISO 16577:2016, 3.108]

3.19
mRNA
messenger RNA
subtype of ribonucleic acid which serves as the template for protein synthesis

3.20
no template control
NTC
control reaction containing all reagents except the extracted test sample template nucleic acid

Note 1 to entry: This control is used to demonstrate the absence of contaminating nucleic acids. Instead of the template DNA, for example, a corresponding volume of nucleic acid free water is added to the reaction. The term "PCR reagent control" is also sometimes used.

3.21
normalization
modification of the measured quantity of a nucleic acid target sequence by subtraction of (C_q scale) or division by (linear scale) quantity or quantities of parameters which reflect non-specific technical factors

3.22
partitions
droplets or chambers of nominally equivalent volume into which dPCR mix of reagents and template is randomly distributed and then amplified by PCR

3.23
PCR assay
assay
qPCR (3.25) or *dPCR* (3.10) measurement method with specified oligonucleotide primers (and, in some cases, a probe or probes) that is used to identify and/or quantify a nucleic acid target

3.24
PCR efficiency
E
fraction of molecules amplified in each PCR cycle

EXAMPLE If a test tube contains 100 target molecules and after one cycle of PCR contains 180 molecules E = 0,8. The calculated rate of amplification is reported as a percentage or a fraction of 1. A 100 % efficiency equates to amplicon doubling during every cycle.

3.25
quantitative real-time PCR
qPCR
enzymatic procedure which combines the in vitro amplification of specific DNA segments with the detection and quantification of specific PCR products during the amplification process

Note 1 to entry: While the PCR is producing copies of the relevant DNA sequence, the fluorescent marker fluoresces in direct proportion to the amount of DNA present (which can theoretically be back-calculated to infer the original amount of that particular DNA present in a sample prior to initiation of PCR).

[SOURCE: ISO 16577:2016, 3.162, modified — The word "quantitative" has been added to the term as well as "quantification" to the definition.]

3.26
reference gene
endogenous gene
gene target present in each sample at approximately constant concentration that is resistant to response fluctuations due to changes in biological or experimental conditions, or stable within a particular species or taxon

Note 1 to entry: Reference genes have, historically, been referred to as housekeeping genes. However, when measuring RNA, many targets can be used which cannot be considered as housekeeping genes; hence the term of preference is now reference gene[1].

3.27
reverse transcription
RT
process of making cDNA from an RNA template, using the enzymatic activity of a reverse transcriptase associated with one or more oligonucleotide primers under a suitable set of conditions

[SOURCE: ISO 16577:2016, 3.180, modified — "DNA" has been replaced by "cDNA".]

3.28
reverse transcription efficiency
RT efficiency
proportion of RNA molecules converted to cDNA expressed as a percentage

EXAMPLE An RT efficiency of 80 % refers to 80 % of RNA templates being converted to cDNA.

3.29
RT minus control
RT(–)
RT-PCR containing test sample template nucleic acid and all the amplification reagents except the reverse transcriptase enzyme

Note 1 to entry: RT minus control is used when quantifying RNA and measures background arising from residual genomic DNA in the sample.

3.30
reverse transcription dPCR
RT-dPCR
process by which an RNA strand is first reverse transcribed into its DNA complement (complementary DNA or cDNA) using reverse transcriptase and the resulting cDNA is amplified using dPCR

Note 1 to entry: This process can be one- or two-step.

Note 2 to entry: In one-step RT-dPCR, RT and dPCR amplification steps are performed sequentially, in the same tube with gene-specific primers.

Note 3 to entry: In two-step RT-dPCR, RT and dPCR stages are performed as two independent reactions. In this case, the RT step can use non-specific primers (i.e. a blend of oligo-dT primers and/or random oligonucleotides) to produce a global cDNA population from all transcripts in the RNA sample. The cDNA is then used for subsequent analysis by dPCR and interrogated for the sequences of interest using gene-specific PCR primers.

3.31
reverse transcription qPCR
RT-qPCR
process by which an RNA strand is first reverse transcribed into its DNA complement (complementary DNA or cDNA) using reverse transcriptase and the resulting cDNA is amplified using qPCR

Note 1 to entry: This process can be one- or two-step as is the case for RT-dPCR.

[SOURCE: ISO 16577:2016, 3.181 modified — Note 1 to entry has been added.]

3.32
sample
small portion or quantity, taken from a population or lot that is ideally a representative selection of the whole

[SOURCE: ISO 16577:2016, 3.185]

3.33
specificity
analytical specificity
ability of a measurement procedure to determine solely the quantity it purports to measure

EXAMPLE PCR assay specificity corresponds to its ability to detect only the intended target and that quantification of the target is not affected by cross-reactivity from related or potentially interfering nucleic acids or specimen-related conditions.

[SOURCE: ISO 15193:2009, 3.9, modified — Example has been added.]

3.34
single nucleotide polymorphism
SNP
single nucleotide variation in a genetic sequence that occurs at appreciable frequency in the population

[SOURCE: ISO 25720: 2009, 4.23]

3.35
single nucleotide variant
SNV
DNA sequence variation that occurs when a single nucleotide, A, T, C, or G, in the genome (or other target sequence) differs between templates

3.36
target sequence
nucleic acid target sequence
specific DNA sequence targeted for detection, e.g. by PCR

[SOURCE: ISO 16577:2016, 3.203]

3.37
template
strand of DNA or RNA that specifies the base sequence of a newly synthesized strand of DNA or RNA, the two strands being complementary

[SOURCE: ISO 16577:2016, 3.206]

3.38
test sample
sample prepared for testing or analysis, the whole quantity or part of it being used for testing or analysis at one time

[SOURCE: ISO 16577:2016, 3.210]

3.39
total nucleic acid
total quantity of nucleic acid in a sample following nucleic acid extraction expressed as mass concentration

Note 1 to entry: Total nucleic acid refers to the expected majority species in a particular extract (i.e. DNA or RNA).

4 Design of measurement procedure

4.1 General

Design of a nucleic acid quantification experiment shall include the selection of appropriate type and number of samples, the process stages for replication, the controls that should be incorporated, the need for randomization of samples and standard arrangement within the experiment. Specific factors to be considered are outlined below. Evaluation of the precision of the assay for the nucleic acid target sequence at the intended test sample range during method validation (8.2) should guide the number of replicate reactions performed during a routine analysis.

4.2 Quantification method

4.2.1 General

The choice of quantification strategy will be dependent upon the application. The major methodological approaches which may be used, together with the requirements for application, are described in 4.2.2 to 4.2.5.

4.2.2 qPCR determination of nucleic acid concentrations using a calibration curve

The calibration curve shall be constructed using independent measurement standards with specified absolute or relative concentrations [e.g. copy number concentration (copies/µl); mass ratio standards (g/kg); International system of units (SI)] that have the same or similar matrix as the test samples.

The imprecision of the calibration curve shall reflect the uncertainty of the measurements and propagate when estimating the measurement uncertainty of test sample concentrations.

If RNA is analysed, the calibration standards shall contain RNA and be pre-treated in the same way as the test samples, i.e. including a reverse transcription step. If circular DNA is analysed, it shall be linearized to remove any supercoiling. If the test sample is single stranded DNA (ssDNA) (e.g. cDNA), the measurement standards should either be single stranded DNA or the calibration curve formula should be amended to reflect the C_q difference of 1 unit as the ssDNA template is not amplified in the first PCR cycle[2].

Calibration solutions shall be evenly spread across the concentration range, and preferably extend beyond it[3]. A minimum of 5 different concentrations should be used, each in at least duplicate.

Concentrations of test samples are estimated from the calibration curve. The concentration (c_i) is estimated using Formula (1):

$$\log_{10} c_i = \frac{C_q - a}{b} \quad (1)$$

where

C_q is the C_q of the test sample;

a is the intercept of the calibration curve;

b is the slope.

NOTE "Absolute quantification" is frequently used by instrument manufacturers referring to the estimation of concentrations of unknown samples by means of a calibration curve. This usage of the term "absolute quantification" is incorrect, as the calibration curve is constructed using calibration solutions of known concentrations.

4.2.3 dPCR determination of copy number concentration using molecular counting

dPCR is an end-point measurement that provides the ability to quantify nucleic acid target sequence without the use of a calibration curve. The dPCR mix containing test solution is randomly distributed into discrete partitions of nominally equivalent volume such that some partitions contain no nucleic acid template and others contain one or more template copies. The partitions are thermally cycled to end-point and then read to determine the fraction of partitions with a positive reaction. Poisson statistics[4] should be used to estimate the target DNA copy number.

The mean number of copies per partition (λ) is calculated using Formula (2):

$$\lambda = -ln\left(1 - \frac{N_P}{N_T}\right) \quad (2)$$

where

N_P is the number of positive partitions;

N_T is the total number of partitions.

Copy number concentration (copies μl^{-1}) in the dPCR ($C_{dPCR\,mix}$) is estimated by Formula (3):

$$C_{dPCR\ mix} = -ln\left(1 - \frac{N_P}{N_T}\right) \cdot \frac{10^3}{V_P} \quad (3)$$

where V_P is the average partition volume (nl).

The partition volume applied in calculation of copy number concentration shall be based on empirical measurements performed during instrument validation or based on published reports, and uncertainty in the measurement of partition volume shall be reflected in the combined uncertainty of the copy number concentration measurement (see 10.4).

If the dPCR mix and prior dilutions of the test samples are prepared volumetrically, copy number concentration (copies μl^{-1}) in the test solution (C) is estimated by Formula (4):

$$C_{dPCR\ mix} = -ln\left(1 - \frac{N_P}{N_T}\right) \cdot \frac{10^3}{V_P} \cdot D \quad (4)$$

where D is the volumetric dilution factor from the test solution to the dPCR mix.

If the dPCR mix and prior dilutions of the test solution are prepared gravimetrically, copy number concentration (copies μl^{-1}) in the test solution (C) is estimated using [Formula (5)] compared volumetric preparation.

$$C_{dPCR\ mix} = -ln\left(1 - \frac{N_P}{N_T}\right) \cdot \frac{10^3}{V_P} \cdot \frac{\left(m_{dPCR\ premix} + m\right)}{m} \cdot \frac{\rho}{\rho_{dPCR\ mix}} \quad (5)$$

where

ρ is the density of the test solution in milligram per µl;

$\rho_{dPCR\,mix}$ is the density of the dPCR mix in milligram per µl;

m is the mass of the test solution in milligram;

$m_{dPCR\,premix}$ is the mass of the PCR premix solution in milligram.

If the template is single-stranded, the estimated copy number concentration in the test solution refers to copy number concentration of single-stranded template. If the template comprises concatemers or tandem repeats of target sequence, the template shall be digested with an enzyme that has a recognition site between the target sequences but not within the target sequence. The digestion step is required so that all target sequences are independent and can be randomly distributed during the partitioning process. Inefficient digestion will result in an underestimate of the target copy number concentration.

4.2.4 Relative quantification by qPCR

Nucleic acid quantification by qPCR data based on C_q values without the use of a calibration curve is often referred to as 'relative quantification'. As C_q values are a logarithmic variable, a delta C_q value essentially expresses a ratio measurement between two quantity values.

The primary delta C_q value calculated in a data analysis workflow should be based on C_q values of two samples measured with the same PCR assay. It is not recommended that the primary delta C_q calculation is based on C_q values of two PCR assays for the same sample as the C_q values do not bear any relationship to each other in terms of target quantity and the independent amplification properties and threshold setting of the different PCR assays make direct comparison irreproducible[1]. In addition, calculation of delta C_q values based on two samples enables the conversion to linear scale (commonly referred to as relative quantities) to take account of PCR efficiency (see 6.2.3) using Formula (6)[5]:

$$\text{Relative quantity} = (1+E)^{\Delta C_q} \quad (6)$$

where

E is the PCR efficiency;

ΔC_q is the delta C_q value.

Due to the absence of a calibration curve, comparison of data from multiple experiments requires the inclusion of an inter-plate calibrator or reference sample which is included in each plate in order to normalize differences in threshold setting[7].

For qPCR measurements of copy number variation whereby gene amplification or deletion is compared to a reference gene[7] a reference sample with known 1:1 ratio is required to normalize for possible differences in qPCR efficiency between the two PCR assays.

In addition, qPCR measurement procedures applying a relative quantification approach should include an additional independent reference sample or reference material control on each qPCR plate in order to monitor the reproducibility of the quantification approach over time, as, unlike with the use of a calibration curve (see 4.2.2), a calibrator with an independently assigned value is not available and PCR efficiency is not measured in each experiment.

Where statistical treatment, including the provision of confidence intervals or expanded measurement uncertainty, is based on a distribution assumption, the relevant assumption should be checked for validity. Such checks may include, for example, a statistical test for departure from an assumed distribution or inspection of a quantile-quantile plot. A minimum of 30 independent data points should be generated for any such check of distribution assumptions.

EXAMPLE An assumption of normality can be checked using a Shapiro-Wilk test or using a normal probability plot.

NOTE Checks on distribution assumptions do not constitute proof of a particular distribution. Rather, they indicate that departures from the assumed distribution are not so large as to cause serious concern for the particular data set.

If statistical analysis is performed with log-transformed data, confidence intervals and measurement uncertainties should be reported on the same scale. If log-transformed values are converted to a linear scale, asymmetric confidence intervals or measurement uncertainties shall be calculated[8], or evidence

provided that a symmetric approximation of the confidence interval on the linear scale is sufficient to cover the largest of the two asymmetric confidence intervals.

4.2.5 dPCR determination of ratio between two targets

To determine the ratio (R) between Target A and Target B within the same test solution, duplex dPCR assays can be used. When analysed under duplex assay conditions, factors for volume, mass, density and dilution cancel out for the two targets and Formula (7) is the resultant measurement formula:

$$R = \frac{ln\left(1 - \frac{N_{\text{P Target A}}}{N_{\text{T}}}\right)}{ln\left(1 - \frac{N_{\text{P Target B}}}{N_{\text{T}}}\right)} \quad (7)$$

where

$N_{\text{P Target A}}$ is the number of partitions positive for Target A;

$N_{\text{P Target B}}$ is the number of partitions positive for Target B.

Further guidance for dPCR-based ratio quantities is provided in References [4], [7].

4.3 Normalization strategy

Quantification methods applying normalization shall demonstrate that the normalization strategy is appropriate.

Normalization should minimize the impact of technical variation, or 'noise', to facilitate determination of true biological variation.

Amplification bias in qPCR-based quantification of miRNA has been reported[9]. This should be taken into consideration for normalization of miRNA measurement results.

Normalization compensates for factors influencing the sample in general as opposed to being specific to the nucleic acid target and therefore avoids sources of non-specific variation confounding measurement of the gene of interest. Technical factors which are controlled for with an effective normalization strategy include sampling variability, sample deterioration during transport and storage and yields of nucleic acid extraction and reverse transcription.

Although normalization is commonly applied to qPCR C_q values, both C_q values and copy number values can be subject to normalization.

There are many methods for normalizing technical differences, which can be appropriate to the analysis of DNA or mRNA (gene expression)[10]. Further guidance for specific normalization strategies is given below.

a) Normalization to reference genes. For quantification of genomic DNA (gDNA), data may be normalized to one or more reference genes. Multiple genomic loci should be evaluated to ensure the chosen marker is representative of the genomic copy number[11]. The reference gene(s) used shall be validated in terms of their stability in sample types.

b) Normalization to expression of reference genes. For gene expression studies, the target quantity should be normalized to multiple reference genes due to the possible variable expression in a single reference gene and consequent bias in analysis of the GOI[1]. Reference genes for measurements of mRNA and miRNA expression shall be empirically validated under the experimental conditions and samples types to which the measurement procedure is applied. It is possible to use statistics to determine the optimum number of reference genes for normalization[12][13][14].

c) Normalization to the expression of all studied genes, often referred to as global normalization. This option can be used when no stable genes to be used as references can be identified (such as in some cases for miRNA expression measurements[15] and for single cell gene expression studies) provided that a sufficiently large total number of targets are measured. The number of targets required for global normalization should be evaluated[16].

d) Normalization to the amount of material used, in cases where the test result of a qPCR- or dPCR-based measurement procedure is reported for a specified input quantity. Appropriate examples would be the number of cells, volume of blood, or total amount of RNA. For certain applications, such as those based on only a few cells where expression is stochastic and the input quantity is independently defined (e.g. by microscopy), this can be better than normalizing to reference genes[17].

4.4 Controls

The qPCR or dPCR method shall have the correct controls to evaluate the overall quality and reliability of the data produced. The nature of the control should accurately reflect the test samples. Methods should specify the positive and negative controls included in each analysis experiment. Examples of appropriate controls are given in Table 1.

Negative controls shall be included alongside test samples, with both PCR set-up controls and negative controls having been subjected to the same extraction and preparation processes as the samples analysed. A number of negative controls should be included and their preparation interspersed with that of the test samples to obtain a representative estimation of the level of contamination in the analytical process. For RT-qPCR or RT-dPCR methods, negative controls shall include RT(−) controls containing positive RNA samples which have been processed with the RNA isolation method to be used for the test samples.

Where applicable, the nucleic acid sequence purity of positive control materials should be verified by deep sequencing. For positive control materials which are used for calibration, the method with which quantity values have been assigned shall be specified.

Table 1 — Positive and negative controls for targeted nucleic acid quantification methods

Type of control	Control name	Typical composition	Reason for inclusion
Negative	NTC	Reaction containing water or buffer instead of nucleic acid template	Detecting contamination of the measurement at the reaction set-up stage (qPCR/dPCR for analysis of DNA; RT-qPCR/dPCR or RT for 1- or 2-step analysis of RNA respectively). Detection of unintended amplification products such as amplified primer-dimers which can occur with the use of dsDNA binding dyes.
Negative	Extraction blank	Extraction containing water or buffer instead of test sample	Control processed alongside test sample to detect of contamination at the extraction stage
Negative	RT(−)	RT reaction without addition of RT enzyme	Amplification of gDNA (for mRNA assay)
Negative	Specificity	Matrix-matched sample or gDNA control not containing the target template	Characterization and monitoring of false positive rate (e.g. 100 % wild-type gDNA for quantification of SNVs; non-modified variety for GMO testing)
NOTE Control categories adapted from ISO 24276.			

Table 1 *(continued)*

Type of control	Control name	Typical composition	Reason for inclusion
Positive	Qualitative	Well characterized biological material or nucleic acid solution	Qualify the functionality of the reaction components and to assess the efficiency of the PCR assay. Assessing reaction specificity for genotyping assays by post-amplification melting curve analysis.
Positive	Quantitative	Well characterized biological material or nucleic acid solution	As qualitative positive control; also assessing the quantitative output of a measurement or for calibration.
Positive	Internal positive control	Sample or matrix blank spiked with exogenous template	Confirmation that no reaction inhibition has occurred (QC for false-negative results).
NOTE Control categories adapted from ISO 24276.			

5 Sample QC — Total nucleic acid quantity, integrity and purity

5.1 General

Pre-analytical procedures and matrix specific nucleic acid extraction shall be carried out as appropriate for the test sample, and in line with relevant ISO standards and guidance documents where available (for example, ISO 20184 series and ISO 20186 series).

Generally total nucleic acid mass concentration (commonly expressed as ng/µl or µg/ml) should be measured in order to ensure consistent sample input per qPCR or dPCR, the performance of which can be affected by variability in the total nucleic acid matrix of the reaction. Analysis of degraded samples can result in poor quality data and inaccurate quantification. In addition the presence of inhibitors can differentially affect dPCR and qPCR[18].

To demonstrate that qPCR/dPCR input is standardised and that nucleic acid samples are sufficiently pure, concentrated and free of components which inhibit or enhance downstream qPCR or dPCR the following methods for characterization of the purified nucleic acid sample shall be described:

— nucleic acid integrity;

— nucleic acid purity.

If one or more of these steps is not applicable or practical, the reason should be stated. For example, in some workflows reaction input would be normalized to the input quantity, for example, volume of biofluid (see 4.3) rather than total nucleic acid quantity. Likewise, if single cell analysis is performed, the quantity of total nucleic acid is too low for analysis of nucleic acid integrity and purity analysis is not applicable.

General methods used for measuring total nucleic acid concentration and assessing its quality are described in 5.2 to 5.4 together with requirements for their application.

5.2 Total nucleic acid quantification

5.2.1 General

Quantification of total nucleic acid (DNA or RNA as appropriate) can be performed using different approaches including ultraviolet spectrophotometry, fluorescent DNA/RNA-binding dyes, and qPCR.

The homogeneity of a nucleic acid sample is a critical factor in any quantification method. The nucleic acid sample shall be completely dissolved and mixed thoroughly prior to quantification. Information

on variation between replicate measurements of the same sample for typical sample types should be provided.

5.2.2 Spectrophotometry

Nucleic acids strongly absorb in the UV with a maximum at or near 260 nm so can be quantified by measuring UV absorption using a spectrophotometer (see Annex A). Consideration should be given to the following sources of bias affecting this technique:

— all nucleic acid species present contribute to absorbance (i.e. DNA, RNA, short oligonucleotides and free nucleotides) therefore the concentration of the nucleic acid of interest (e.g. DNA) can be overestimated;

— many chemical compounds absorb at or near 260 nm (e.g. phenolic solutions used for isolation or lysis), representing another potential source of positive bias.

Calibration with a certified reference material with composition sufficiently similar to that of the test sample is required for mass concentration results to be traceable to the SI.

5.2.3 Fluorometry

The use of fluorescent dyes to quantify total nucleic acid is an alternative quantification strategy. Fluorescent methods are dependent on the fluorescence characteristics of small molecules or dyes upon binding to nucleic acid. Consideration should be given to potential sources of bias affecting fluorometric methods including:

— denaturation state of nucleic acid;

— nucleic acid degradation as some dyes preferentially bind to molecules above a certain size;

— temperature;

— pH;

— exposure to UV light (photobleaching);

— chemical contaminants which can affect binding efficiency (supplier information should be checked);

— chemical contaminants which can cause fluorescence quenching as this significantly affects fluorometric readings. Heavy ions such as iodide anions or cesium cations as well as neutral molecules like acrylamide can act as quenchers.

Measurements are relative to a calibration material or standard nucleic acid solution therefore measurement accuracy depends on the accuracy of the value assigned to the calibration material and the similarity in composition between the test sample and the calibration material. Evaluation of positive and negative controls should also be included.

5.2.4 Assessment of total DNA concentration using qPCR/dPCR

qPCR and dPCR can be used to quantify total DNA of a specific taxonomic assignment, e.g. total human DNA, total bacterial DNA, total fungal DNA by using primers of the corresponding taxonomic specificity combined with conversion to mass concentration using an estimate of the genome size of the organism. This approach measures the quantity of amplifiable DNA, not necessarily the total DNA concentration in a sample, therefore results should be interpreted together with information on the integrity (5.3) and purity (5.4) of the sample.

The following requirements apply to qPCR/dPCR based methods when applied to total DNA quantification.

— Specificity: Evidence of PCR assay specificity shall be provided, including for commercial PCR assays. For species-specific primers the absence of homology with other species shall be demonstrated. For primers and probes of known sequences, specificity shall be validated according to 6.1.4 and 6.2.5.

— Metrological traceability: For traceable quantification of total gDNA copy number or mass concentration, the calibration curve shall be based on a reference material, characterized for mass concentration and the number of genomic copies per volume unit. The source of the calibration material shall be stated.

— Commutability of the calibrant: the PCR efficiency of the calibrant should be the same as the PCR efficiency of the sample analysed.

— Absence of PCR inhibition: absence of inhibition in the analysed sample (see 5.4).

— The amplicon size of the PCR assay for measurement of total DNA concentration shall be appropriate for the intended downstream qPCR/dPCR analysis, i.e. similar in amplicon size or accompanied with evidence of nucleic acid integrity.

5.3 Nucleic acid integrity

Nucleic acid integrity impacts on accurate quantification of specific target sequences and information regarding sample integrity shall be provided.

Methods which are commonly used to assess the integrity of the nucleic acid sample with example applications are described in Annex B. The most commonly used methods are gel electrophoresis (capillary or slab) and measurement of differentially sized amplicons. Consideration shall be given to positive control and calibration materials which are used to accept/reject the integrity of nucleic acid in the test sample or quantify its integrity. Examples could include a high-quality sample as positive control for gel electrophoresis or intact plasmid template for 5′/3′ ratio or differential amplicon size assay. Additionally, performance of the downstream qPCR or dPCR measurement procedures with test samples of variable integrity should be characterized in order to set criteria for nucleic acid integrity.

5.4 Nucleic acid purity

Nucleic acid purity shall be determined. This may include, but is not limited to a check for:

— absence of interfering chemical impurities: organic solvent, polysaccharide and protein components, carried through from the sample matrix or extraction step;

— absence of contaminating gDNA in an RNA sample (optional for presence of RNA in a DNA sample).

Different approaches may be applied including, but not limited to:

a) UV Spectrophotometry. The presence of potentially contaminating organic substances can be crudely determined by measuring the absorbance readings at 280 nm and 230 nm for proteins, and at 230 nm for chaotropic salts and phenol. Critical parameters are the ratio of absorbance at 260 nm to 280 nm and the ratio of absorbance at 260 nm to 230 nm (see Annex A). This approach is limited to samples with sufficient concentration for total nucleic acid quantification by UV spectrophotometry (see 5.2.2).

b) qPCR and dPCR assay with internal control for PCR amplification. An increase or decrease in the C_q of an exogenous molecule spiked into the sample in comparison to the C_q value for the equivalent amount of exogenous molecule spiked into buffer indicates the presence of reaction inhibitors or enhancers, respectively[19]. Conversely for dPCR or calibration curve-based qPCR method, an increase or decrease in the copy number of an exogenous molecule spiked into the sample in comparison to the copy number measured for the equivalent amount of exogenous molecule spiked into buffer indicates the presence of reaction enhancers and inhibitors, respectively.

c) Fluorometric methods with specificity for RNA or DNA (see 5.2.3).

d) RT(−) control reactions or assays targeting gDNA are also suitable to determine gDNA contamination in an RNA sample (see 6.3.2).

Applications of methods for analysis of nucleic acid purity to a range of clinical samples are provided in Reference [20].

6 Assay design and optimization for quantification of nucleic acid target sequences

6.1 Assay design

6.1.1 General

Requirements for RNA and DNA quantification by dPCR/RT-dPCR and qPCR/RT-qPCR assay design are listed below.

NOTE Reference [21] can also offer guidelines.

6.1.2 Amplicon selection

Shorter amplicons are typically amplified with higher efficiency than longer ones but should be long enough to be sufficiently differentiated from potential primer dimers when fluorescent dye detection is used. An amplicon size of 250 bp or less is recommended for the majority of dPCR and qPCR assays. GC rich areas, repetitive regions, and regions with commonly known SNPs should be avoided unless differential detection of SNPs is intended[22]. The sequence between primers should allow an efficient and specific site for probe binding if a probe based detection chemistry is used.

NOTE The Single Nucleotide Polymorphism database (dbSNP) is a curated database of commonly known SNPs in the human genome. See Reference [23].

6.1.3 Primer and probe design

Primers should be designed with an annealing temperature between 50 °C and 68 °C and should possess 40 % to 60 % GC content while lacking any significant secondary structures[22]. Optimally, the difference between annealing temperatures of the primer pair should be 1 °C to 2 °C.

Probe length should be < 30 bases. When using a hydrolysis probe, the annealing temperature of the probe should be at least 5 °C higher than that of the primers. Additionally, guanine at the 5' end of a probe should be avoided due to the possibility of quenching the fluorescence signal. The melting temperature of the probe can be increased by use of minor groove binders or modified nucleotides (such as locked nucleic acids).

6.1.4 In silico evaluation of specificity

PCR assays shall be designed to ensure specificity and specificity shall be checked in silico.

PCR primers and probes designed to a target sequence can also bind to similar sequences (for example, homologous genes within the same genome) if the sequences match or differ by only a few base pairs to the non-intended target. If the region between the primer pair is sufficiently small (<500 bp), an amplicon can be produced during PCR. In this case, it could be that the assay is not specific for the gene target as the non-intended template could contribute to the reporter signal if present. The addition of a probe to an assay design can improve specificity.

In order to evaluate specificity to target amplicon sequence, primers and probes shall be screened against the reference database transcript or genome database for the intended templates and the database(s) of possible contaminating templates (e.g. using Reference [24]). In the case of a potential

non-intended PCR product being identified by the initial in silico screen, the homology of the probe sequence to the possible amplicon produced by the non-specific binding of the primer pair shall be evaluated (e.g. in terms of number of mismatches) to inform the specificity of a probe-based assay.

miRNAs often form families of closely related sequences which can differ by only 1 bp to 2 bp, therefore miRNA assay specificity shall be checked in silico against other members of the same miRNA family in order to predict degree of cross-reactivity and inform in vitro testing (see 6.2.5). The specificity of the assay to the mature miRNA compared to its primary and precursor miRNA forms should also be verified.

NOTE The miRBase database[25] is a searchable database of published miRNA sequences and annotation which provides information on miRNA sequences and families. miRBase versions after miRBase16 can contain false miRNAs that have been identified through NGS due to small RNA fragments in library preparation[26].

6.1.5 RT-qPCR/RT-dPCR design

For RNA quantification, the effect of contaminating gDNA in the test sample should be minimised by selecting regions which are unique to the RNA sequence where possible.

EXAMPLE For mRNA targets which undergo splicing of intronic sequences, the RT-qPCR or -dPCR assay can be designed using primers that span exon-exon boundaries.

In silico specificity analysis should include a BLAST search against the specific genomic reference sequence. The impact of cross-reactivity should be characterized during specificity testing (see 6.2.5) and method optimization with the test sample (see 6.3.2).

For RT methods which use oligo-dT based priming for synthesis of cDNA, a 3' bias can occur due to fragmentation of the mRNA transcript or reduced processivity of the RT enzyme towards the 5' end of the molecule. Therefore, the location of the amplicon within the transcript should also be taken into account.

6.2 Assay optimization using purified samples

6.2.1 General

Nucleic acid quantification assay performance shall be optimized. Requirements for method parameters that should be considered to obtain optimised assay performance, including signal output, PCR efficiency and specificity are listed below.

Nucleic acid quantification-based assays can be purchased from a number of commercial providers. Assays should be validated by each laboratory before they are used for analytical purposes. Suppliers' claims relating to the performance of assays shall be verified in-house and further assays may be necessary to obtain optimized assay performance.

The ideal positive control materials (see 4.4) for initial assay optimization (see 6.2.2, 6.2.3, 6.2.4) are simple template molecules (e.g. plasmid, oligonucleotide constructs, in vitro transcribed RNA) containing only the target region of interest. miRNA assay optimization can be performed using synthetic RNA oligonucleotides of the same sequence. These are complementary to positive control materials of greater nucleic acid complexity (e.g. gDNA, total RNA from cell lines) which contain possible cross-reactive sequences (e.g. pseudogenes; transcript isoforms) in enabling specificity to be evaluated (see 6.2.5).

6.2.2 Optimization of fluorescence signal

Initial optimization of a qPCR or dPCR assay shall include testing a range of PCR annealing temperatures, PCR cycle numbers and primer/probe concentrations to determine the optimal conditions for fluorescent signal output.

In the case of dPCR, optimization is also required to minimize the number of partitions with an intermediate fluorescence amplitude (and C_q for real-time dPCR systems) between the negative and

positive clusters. The resolution of a digital assay (Rs) is a quantitative measure of how well the two populations (positive and negative) can be differentiated in a linear separation. It is defined as the difference in fluorescence between the two peaks, divided by the combined widths of the peaks. A resolution of 2,5 has been proposed to allow for a certain amount of deterioration of the resolution in more difficult samples[27].

6.2.3 (RT)-qPCR amplification efficiency

Once basic assay parameters have been optimized (see 6.2.2), the PCR efficiency of a qPCR- or RT-qPCR based measurement procedure shall be determined.

PCR efficiency shall be evaluated by analysis of a calibration or dilution series of DNA (qPCR) or RNA (RT-qPCR) with the assay (see Annex C). For RT-qPCR, an RNA calibration or dilution series shall be performed as this enables validation of equal RT efficiency over the applicable linear range of the assay[28]. For a RT-qPCR assay, PCR efficiency with a DNA calibration or dilution series may also be analysed to gather information on the PCR efficiency of the primers independent of the RT step.

The slope of the calibration curve or dilution series can be translated into an efficiency value (see Annex C). The efficiency of the (RT)-qPCR should be evaluated in multiple experiments (minimum of 3) and a confidence interval calculated to assess the precision of the estimate. In absence of interference (see 6.3.1), mean PCR efficiency should be between 90 and 110 % (−3,6 ≥ slope ≥ −3,1). The degree of linearity shall be characterized in terms of correlation coefficient (e,g, Pearson's correlation coefficient, R). The correlation coefficient R^2 should be greater than 0,99.

NOTE In theory, PCR efficiency cannot exceed 100 % as the number of template molecules cannot be more than doubled in each cycle. However, observed PCR efficiency calculated by regression analysis can exceed 100 % by chance. If observed PCR efficiencies repeatedly exceed 100 %, this can be due to the presence of inhibiting substances (see 6.3.1) or an artefact in the standard curve samples or data analysis parameters (see Annex C).

6.2.4 RT efficiency

RT efficiency can be highly variable[29][30][31] therefore RT efficiency shall be evaluated during assay optimization. Evidence of optimization of RT efficiency may include:

— comparison of alternative RT enzymes/kits[31];

— quantification of RT efficiency using in vitro transcribed RNA templates[30][32].

Due to the short length of miRNA and siRNAs, methods targeting these molecules typically incorporate a modified RT step involving elongation of the miRNA molecule or stem loop primers in order to extend the target sequence to a length which is suitable for PCR amplification. The impact of miRNA RT strategy on RT efficiency and assay specificity (see 6.2.5) can be assessed by comparison of alternative approaches during method optimization.

6.2.5 Specificity

The specificity of an assay to the intended target and potential cross-reactivity with homologous sequences (e.g. SNPs/SNVs, pseudogenes, paralogues or orthologues sequences) which can be present in the typical biological sample shall be evaluated. For assays targeting eukaryotic genomic regions, the specificity of an RT-qPCR or RT-dPCR for the intended RNA transcript should also be evaluated by testing with a gDNA sample. miRNA assay specificity shall be tested against synthetic RNA oligonucleotides of closely related miRNAs. In silico analysis of primer/probe specificity (see 6.1.4) can inform wet lab testing.

Measurements of positive control materials (see 4.4) should be compared with measurements of similar input quantities of closely related templates which are likely to be present in the sample. Modification of PCR annealing temperature may be required to improve the specificity of the assay (see 6.2.2).

Melting curve analysis may also be performed as evidence of primer specificity. Melting curve analysis is a post-PCR analysis performed to evaluate the specificity of amplified products based on their melting

characteristics. Reactions performed in the presence of dsDNA binding dyes are incubated through a range of increasing temperatures. For detection of a single nucleic acid target sequence, a single peak should be observed in the derivative plot as evidence that a single amplicon has been produced in the reaction.

NOTE The melting temperature (T_m) is dependent upon the length of the DNA sequence, G:C content, buffer, and sequential arrangement of nucleotides.

The false positive rate of the assay expressed as a proportion (%) of the target template concentration or quantity per reaction shall be calculated for the assay. The false positive rate is required for establishment of the LOD (see 8.4).

6.3 Method optimization using test samples

6.3.1 Effect of PCR inhibitors in sample matrix

Nucleic acid samples that contain inhibitory compounds (e.g. sample preparation reagents, excessive protein) can lead to partial or complete inhibition of downstream PCR. The presence of PCR inhibition can also be indicated by sporadic or late amplification, poor repeatability and nonlinearity of the calibration curve. In addition to assessment of major chemical or nucleic acid impurities described in 5.4, the influence of the sample matrix on the specific qPCR/dPCR assay shall be evaluated, as differential susceptibility of assays to inhibitors has been observed[33].

Requirements to measure the impact of the sample matrix on assay performance and assess specific PCR inhibition include, but are not limited to:

— Quantification of an internal PCR control bearing the target nucleic acid sequence, such as control amplicon, plasmid, purified gDNA. The presence and extent of inhibition (or enhancement) may be determined by preparing control reactions containing:

  a) the internal PCR control added to extracted sample matrix;

  b) the internal PCR control only (i.e. in the absence of extracted sample/matrix);

  c) extracted sample matrix only (i.e. no internal PCR control) in order to measure the background concentration of the nucleic acid target (if present).

  A minimum of three replicates of each control reactions should be prepared in order to test for statistical significance using an appropriate test (e.g. *t*-test). In the absence of modification of assay performance by the sample matrix, the target nucleic acid quantity measured by Control 1 equals the sum of the quantities measured by Controls 2 and 3, whereas if the assay is inhibited (or enhanced) by the sample matrix the quantity in Control 1 will be significantly less (or more) than the sum of the quantities measured by Controls 2 and 3.

— Sample dilution. As dilution reduces the concentration of potential inhibitors, expected fold differences in concentration or C_q values based on the dilution which match the observed differences provide evidence for lack of inhibition (or enhancement).

— Analysis of individual reaction kinetics[34] and/or the calculation of amplification efficiency using a dilution series[35][36].

6.3.2 Presence of nucleic acid contaminants in test sample

The susceptibility of an RT-qPCR or RT-dPCR assay to gDNA contamination (carried over from the extraction stage) shall be evaluated as described in 6.1.5 and 6.2.5. The presence of contaminating gDNA in the test sample should be identified using RT(−) controls (see 4.4). gDNA contamination should be avoided using DNAse during RNA preparation.

RT(−) controls should show no amplification. However, if RT(−) reactions give a positive result (or a sporadic detection pattern is observed with a proportion of reactions generating signal), their

existence shall be justified and the difference in C_q or concentration between test samples and paired RT(−) reactions characterized. The influence of gDNA-derived signal shall be taken into account in the quantification of the target nucleic acid and calculation of the limit of detection (LOD) of the method (see 8.4).

6.3.3 Validated measurement range

The validated measurement range for the method which encompasses the LOD (see 8.4) should be described and the linear range of the method specified (see 8.5).

In order to establish the validated measurement range of a method, it is necessary to study the response of reference materials (or where reference materials are not available, in-house samples or spiked samples) whose concentrations span the range of interest and reach a limiting dilution.

For quantification of concentration by qPCR using a calibration curve (see 4.2.2), the amount of target nucleic acid in the test samples should fall within the concentration range covered by the calibration curve.

For dPCR, the validated measurement range shall be specified as a range of copies per reaction (or lambda values) as the standard error to the Poisson estimate of concentration (see 4.2.3) varies with the fraction of positive partitions[37][38].

6.4 No template controls

All NTC and negative process controls should show no evidence of PCR amplification. Any non-negative results (e.g. primer-dimer for methods using intercalating dyes) shall be justified with evidence (e.g. using melt curve analysis in 6.2.5).

7 Data quality control (QC) and analysis

7.1 General

Data analysis includes an examination of the raw data, an evaluation of their quality and reliability, fluorescence threshold setting and pre-processing of exported raw data.

7.2 Acceptance criteria

7.2.1 qPCR

qPCR amplification plots usually show a response curve that is sigmoidal corresponding to three amplification phases:

a) amplification of template which is below the detection limit for fluorescent signal;

b) exponential amplification evidenced by a linear slope vs. cycle number; and

c) a plateau where reaction components become limiting; it is possible that the latter is not present in test samples where phase b) is evident at a late cycle number.

Measurement procedures should include evaluation of amplification curves of test sample measurements for evidence of the aforementioned phases of amplification and comparison of the baseline and plateau phase fluorescent signals with those of positive and negative control samples (see 4.4).

7.2.2 dPCR

For chip-based dPCR, image analysis shall display even dispersion of passive reference dye across the partitions and no air bubbles within the chip. Additionally, the random distribution of positives and negatives shall be visually confirmed.

For real-time dPCR, the real-time amplification plots should show reproducible log-linear amplification within a narrow C_q window which relates to the lambda value for the reaction (e.g. C_q values for reactions with 1 and 2 molecules/partition would be expected to be separated by 1 C_q unit[39][40]).

For droplet dPCR, the minimum number of accepted droplets should meet the manufacturer's recommendation or user-defined values. Quality criteria related to the fluorescence amplitude of droplets should include:

— In a plot of droplet fluorescence amplitude against droplet number, streaks of droplets with a fluorescence amplitude either below the negative droplet cluster or above the positive droplet cluster should not be present, as these can indicate anomalies in droplet size.

— In a plot of droplet fluorescence amplitude against droplet number for replicate dPCR assays, there should be minimal shift in fluorescence amplitude of the positive and negative droplet clusters between replicates.

— The proportion of droplets with intermediate fluorescence (between the negative and positive droplet clusters) should be minimised during assay optimization (see 6.2). The proportion of droplets with intermediate fluorescence should be monitored and factored into the uncertainty of the measurement (see 10.4).

7.3 Threshold setting

7.3.1 qPCR

The threshold should be set:

— sufficiently above the background fluorescence baseline to be confident of avoiding having any amplification curve cross the threshold prematurely due to background fluorescence;

— as low as possible to ensure that the threshold crosses at the log-linear phase of the amplification curve where it is unaffected by the plateau phase;

— at a fixed level for all samples measured with the same assay on the same plate.

7.3.2 dPCR

Positive and negative control materials (see 4.4) of high sequence purity are critical for dPCR threshold setting and shall be integrated into the workflow for threshold setting and categorization of partitions with intermediate fluorescence (so called 'rain'), for which multiple approaches exist[41][42][43].

Particularly for competing duplex analysis (e.g. SNP quantification where a single primer pair and ≥ 2 probes are employed, leading to a reduction in fluorescence in double positive droplets), thresholds should be set (or checked if automatic threshold setting is performed) by viewing amplification plots in 2D mode[44].

7.4 Data pre-processing

7.4.1 qPCR using calibration curve

Acceptable limits for the slope of the calibration curve should be specified based on the confidence interval determined for PCR efficiency during assay optimization (see 6.2.3).

7.4.2 Relative quantification (qPCR)

A workflow for pre-processing of C_q raw data shall include compensation for variation in threshold setting between runs [inter-plate calibration (see 4.2.4)].

Assay efficiency shall be corrected for in the calculation of relative quantities[5] (see 4.2.4).

The complete workflow of pre-processing steps for a relative quantification approach depends on the application. For example, in single cell expression studies, target transcripts are often heterogeneously expressed and absent in a proportion of single cell samples, which requires approaches for handling missing data[6].

7.5 Identification of outliers

Data should be inspected for outlying values, for example using Dixon's or Grubb's test. Where there are multiple outliers, checks designed for multiple outlier detection should be used.

NOTE ISO 16269-4 gives guidance on the detection and treatment of outliers.

8 Nucleic acid quantification measurement method validation

8.1 General

The nucleic acid quantification measurement method (qPCR and dPCR) shall be validated. ISO 5725-1 gives general guidance on method validation. Method performance parameters should be provided to give evidence that the method produces results that are suitable for the intended purpose with acceptable level of measurement uncertainty.

Method performance parameters can include specification for:

— specificity (requirements outlined in 6.2.5);

— precision;

— LOQ;

— LOD;

— trueness;

— linearity;

— robustness.

Requirements for characterizing these performance attributes for both qPCR and dPCR measurement procedures are given in 8.2 to 8.7, with specific considerations for qPCR and dPCR provided in 8.8 and 8.9 respectively.

8.2 Precision

Precision is a measure of the variability in independent measurement results obtained for the same sample under stipulated conditions. Depending on the stipulated conditions, measurement precision can be divided into method repeatability, intermediate precision and reproducibility. Within the frame of a single laboratory validation, both the method repeatability and intermediate precision shall be determined.

The number of replicate measurements (within experiment) and independent experiments shall be sufficient to provide a reliable estimate of the standard deviation (SD). ISO 5725-1 gives guidance on the number of replicate measurements to meet a specified uncertainty in the estimation of the SD.

A one-way analysis of the variance (ANOVA) may be performed to calculate the relative repeatability ($s_{\text{repeat,rel}}$) and relative run-to-run variation ($s_{\text{run,rel}}$) standard deviations according to Formulae (8) and (9) respectively:

$$S_{\text{repeat,rel}} = \frac{\sqrt{MS_{\text{within run}}}}{\bar{c}_{\text{sample,meas}}} \tag{8}$$

$$S_{\text{run,rel}} = \frac{\sqrt{\dfrac{MS_{\text{between run}} - MS_{\text{within run}}}{\bar{n}_{\text{repli}}}}}{\bar{c}_{\text{sample,meas}}} \tag{9}$$

where

$MS_{\text{within run}}$	is the within run mean of squares calculated by one-way ANOVA;
$MS_{\text{between run}}$	is the between run mean of squares calculated by one-way ANOVA;
$\bar{n}_{\text{repli}}$	is the average number of replicates per run;
$\bar{c}_{\text{sample,meas}}$	is the average measured sample copy number concentration over all runs.

8.3 LOQ

The precision associated with the LOQ shall be specified; for example, as a SD or relative SD. The LOQ is calculated from the analysis of replicates of sample concentrations in the lower part of the linear range of the measurement procedure.

The precision of LOQ estimates depends on the number of replicates performed at each concentration and the increment in concentration between the samples. A minimum of 10 replicates should be performed at each concentration with small increments in concentration (twofold above and below the expected concentration for the LOQ)[45][46][47].

8.4 LOD

Estimation of LOD shall take account of both the statistical distribution of false-positive results and true positive samples[48]. The probability of false positive detection (β) and false negative detection (α) (for example $p = 0{,}05$) or confidence of classifying a true positive (equal to 1 – α) or false negative (equal to 1 – β) shall be specified.

The false positive rate of the measurement procedure shall be characterized as part of evaluation of specificity (see 6.2.5) and analysis of negative controls (see 4.4, 6.3.2 and 6.4.) In applications where the LOD is primarily determined by the false positive rate of an assay, the statistical distribution of true positive results may be modelled theoretically. For example, the false positive rate with wild-type DNA as the template is critical for the definition of the LOD of a dPCR assay measuring a SNV, therefore the distribution of true positives is modelled theoretically[49].

The true positive detection rate should be estimated from replicate measurements of samples containing target quantities at the lower end of the working range of the measurement procedure, where > 0 % and < 100 % of reactions are positive and cover the range for the required confidence level. For example, at 95 % confidence, the LOD is the lowest concentration where 95 % of the replicates are positive. Calibration materials or positive samples for which the quantity value and measurement uncertainty have been assigned should be used to characterize the true positive detection rate. If dilutions are performed (volumetric or gravimetric), the precision of the dilutions should be characterized and used to calculate the measurement uncertainty of the concentration established as the LOD.

The fraction of positive replicates is plotted versus the concentration of the calibration materials and can be interpolated to obtain better precision by fitting the data to the sigmoidal curve and the confidence interval of the LOD estimate is obtained[47]. Precision of the LOD estimate depends on the number of replicate samples at each concentration and the increment in concentration between samples. A minimum of 10 replicates per concentration level should be performed. Concentration increment should be maximum twofold.

NOTE If a laboratory does not know where to expect the LOD a good strategy is to estimate it first performing a pilot study with just few replicates at each concentration and covering a wider concentration range and then follow up with a large number of replicates over a narrow concentration range.

8.5 Linearity

The linear range of the measurement procedure shall be documented.

The degree of linearity within this range shall be characterized in terms of slope and correlation coefficient (for example, Pearson's correlation coefficient, R) by regression analysis of expected nucleic acid quantity vs. observed nucleic acid quantity. The measurand shall be the quantity measured by the method (see 4.2), rather than an intermediate quantity (for example, C_q) subject to further data processing (see 7.4).

Samples used for the evaluation of linearity shall be representative of typical test samples and may be calibration materials or a dilution series of a test sample. For a dilution series, the relative quantity shall be analysed.

The expected slope of a plot of observed vs. expected value is 1,0, with intercept (0,0). A slope between 0,95 and 1,05 is recommended. The correlation coefficient R^2 should be greater than 0,99.

NOTE Further information on the determination of linearity in dPCR can be found in Reference [50]. Additional information on the use of the correlation coefficient for linearity verification can be found in Reference [51].

8.6 Trueness

Measurement trueness is an expression of how close the mean of an infinite number (i.e. a large number in reality) of results produced by the method comes to a reference value. There are three general approaches to obtain a suitable reference value:

a) use of certified reference materials;

b) recovery experiments using spiked samples; and

c) comparison with results obtained from another method[52].

For a), the certified reference material shall have a very similar matrix to that of the test sample and a target copy number concentration within the same range as the routine samples.

8.7 Robustness

During the robustness test, the effect of small deviations in relevant method parameters on the method performance and the measurement results should be investigated. Relevant method parameters that are likely to influence the method outcome are the concentration and source (manufacturer) of primers and probes, composition of PCR reagents, thermal cycler[53] and thermal cycling parameters.

It is generally considered that the dPCR method, being an end point PCR method, is more robust than qPCR methods, however fluorescence amplitude and annealing temperature are important parameters in discriminating between positive and negative partitions, therefore concentration and source (manufacturer) of primers and probes and annealing temperature should be varied during robustness testing.

8.8 Specific considerations for qPCR method validation

8.8.1 Repeatability of qPCR- or RT-qPCR

Repeatability of a qPCR- or RT-qPCR based measurement procedure should be expressed consistently with the quantification strategy employed: for an approach using a calibration curve (see 4.2.2), the standard deviation (SD) of the concentration units of the calibration curve are appropriate while for a relative quantification approach (see 4.2.4), the SD shall reflect the final quantity calculated by the measurement procedure.

To express repeatability as the coefficient of variation of C_q values (CV_{C_q}), Formula (10) may be used[47]:

$$CV_{C_q} = \sqrt[2]{(1+E)^{\left(SD_{C_q}\right)^2 \times \ln(1+E)} - 1} \qquad (10)$$

where SD_{C_q} is the standard deviation of C_q values.

It is incorrect to express CV_{C_q} by division of the SD_{C_q} by the mean C_q value. The value for C_q repeatability provided by Formula (10) shall be used to calculate the repeatability in the final nucleic acid quantity measured by the measurement procedure.

Measures of precision are often symmetric around the estimated concentration when expressed on the logarithmic C_q scale. If converted to a linear scale the precision measure becomes asymmetric around the estimated concentration. Therefore, it should be considered whether a symmetric approximation of the precision measure on the linear scale is sufficient to cover the positive and negative intervals expressed on a logarithmic scale.

8.8.2 Intermediate precision and reproducibility of qPCR- or RT-qPCR

For a relative quantification approach (see 4.2.4), the precision between independent experiments or between laboratories shall not be expressed as the SD of raw C_q values, as C_q values are not comparable between experiments due to variation in threshold setting. Precision should be expressed for the final quantity of the measurement procedure following data processing (see 7.4) and normalization (see 4.3).

8.9 Specific considerations for dPCR method validation

The precision of dPCR is known to vary with the λ values due to its reliance on Poisson statistics to account for partitions with multiple occupancy[37]. Therefore, dPCR precision shall be established as a range of λ values as well as the quantity measured by the measurement procedure (see 4.2) of the target nucleic acid. In addition for multiplex assays, the λ value range for the nucleic acid templates targeted by the other assay(s) within the multiplex should be specified as this can influence the method performance.

9 Nucleic acid quantification measurement traceability and comparability

9.1 Metrological traceability

The traceability of a measurement result refers to metrological traceability. It relates the result to SI units or other agreed standards/references. Traceability is essential for comparability of analytical results (see ISO/IEC 17025).

Metrological traceability for dPCR and qPCR measurement results may be established in accordance with ISO 17511.

Quantities with the character of a count, including copies of a particular nucleic acid sequence, have the unit one. The unit one is by nature an element of any system of units. Quantities with the unit one can

be considered as traceable to the SI[54]. Formal traceability to the SI can therefore be derived through appropriate, validated measurement procedures.

As an enumeration-based measurement procedure, dPCR can form the basis of a primary reference measurement procedure for nucleic acid copy number concentration (for nucleic acid fragments amplifiable under the given experimental PCR conditions), subject to the establishment of specificity and completeness of count and a statement of measurement uncertainty[55][56].

9.2 Use of reference materials

Reference materials should be used to ensure analytical traceability of the measurement results and to verify the performance of the nucleic acid measurement process. When available, an appropriate nucleic acid-based reference material should be used for its intended purpose.

9.3 Instrument calibration

Instruments used for nucleic acid quantification shall be calibrated using appropriate calibrators and/or reference materials where available. Auxiliary equipment, including but not limited to, balances and micropipettes shall also be calibrated.

10 Measurement uncertainty (MU) in qPCR and dPCR measurements

10.1 General requirements for MU calculations

Measurement uncertainty can be defined as an estimated range of values within which the true value of the measurement resides. The range of values gives an indication of the reliability of a measurement result. The uncertainty estimate includes the effect of both random and systematic errors in the measurement procedure. In the assessment of factors contributing to the measurement uncertainty of a result, all possible sources of variability in the measurement process should be considered. Annex D provides an overview of sources of uncertainty in qPCR and dPCR measurements. Experimental data and other sources of information such as published results and calibration certificates shall be evaluated to create an uncertainty budget which reflects the key analytical steps influencing the measurement result. Further guidance for the calculation of measurement uncertainty is provided in Reference [57] for qPCR and in Reference [58] for dPCR.

Measurement uncertainty calculations for nucleic acid quantification methods should include estimates of:

— repeatability, intermediate precision and reproducibility of qPCR or dPCR measurements;

— bias of qPCR or dPCR measurements;

— uncertainties of values assigned to calibration materials;

— uncertainties of dilution of test samples or calibration materials (random errors in dilution can be captured in intermediate precision if replicates performed throughout the whole process);

— uncertainties related to normalization: measurement uncertainty of reference gene(s) or other genes applied for normalization (see 4.3) shall be combined appropriately according to the formula used to calculate the normalized quantity of the target of interest (for example, geometric mean of multiple reference gene quantities);

— other factors which can reasonably be expected to influence the measurement result.

Uncertainties shall be combined in accordance with ISO/IEC Guide 98-3. The coverage factor applied to calculate an expanded uncertainty with a specified level of confidence (e.g. 95 %) should take into account the number of replicates performed, which determines the degrees of freedom used to calculate the coverage factor.

Experiments performed in method validation can provide information on precision (see 8.2) and trueness (see 8.6) which can be used in calculating measurement uncertainty.

Based on repeatability and between-run estimates (see 8.2), the relative standard uncertainty of the method intermediate precision ($u_{precision,rel}$) associated with the average measured sample copy number concentration can be calculated using Formula (11)[58].

$$u_{precision,rel} = \sqrt{\frac{{s_{repeat,rel}}^2}{\overline{n}_{repli} \times n_{run}} + \frac{{s_{run}}^2}{n_{run}}} \quad (11)$$

where n_{run} is the number of experiments performed during validation.

If the concentration of routine samples is determined by calibration to a reference material (4.2.2), the uncertainty of the certified concentration of the reference material shall be included in the combined uncertainty.

Where an independent reference material is available for evaluation of trueness (8.6), the uncertainty of the bias ($u_{bias,rel}$) can be calculated using Formula (12).

NOTE This approach is only valid in cases where no significant bias is observed or bias is corrected for in reported nucleic acid quantity.

$$u_{bias,rel} = \sqrt{{u_{precision,rel}}^2 + {u_{cert,rel}}^2} \quad (12)$$

where $u_{cert,rel}$ is the relative uncertainty associated with the certified copy number concentration.

10.2 qPCR measurement uncertainty

qPCR measurement uncertainty shall also take into account uncertainty in PCR efficiency. PCR efficiency is intrinsic to the calculation of the nucleic acid target quantity using calibration curve-based approach (see 4.2.2), therefore the variation in PCR efficiency can be captured in intermediate precision data from method validation. For a relative quantification approach (see 4.2.4), PCR efficiency confidence intervals from assay optimization (see 6.2.3) can be used and shall reflect PCR efficiency estimates from multiple experiments (≥3).

10.3 Ratio-based measurements

For ratio-based quantities (see 4.2.4 and 4.2.5), measurement uncertainties shall take account of correlation between variables in accordance with ISO/IEC Guide 98-3:2008, 5.2. If a ratio between two quantities (such as the copy number or C_q of a GOI and C_q of reference gene(s)) is calculated using averaged values for each quantity, an adjustment for correlation is required. However, if a ratio is calculated directly for each replicate measurement (for example, % of SNV/total copies of the SNV-containing region in a single reaction), an adjustment for correlation is not necessary.

Further guidance for the calculation of type A precision contributions to measurement uncertainty for ratio-based dPCR measurements (see 4.2.5) is provided in Reference [7].

10.4 dPCR measurement uncertainty

dPCR measurement uncertainty shall take into account experimental sources of variation; it is not sufficient to calculate measurement uncertainty purely on theoretical 95 % confidence intervals of the Poisson count[59]. As Poisson estimation of the nucleic acid count is intrinsic to dPCR quantification (see 4.2.3), experimental replication [e.g. performed in validation studies (see 8.2)] captures this

source of uncertainty and separate allowance for theoretical precision is not normally required in the contribution of overall precision to measurement uncertainty [see Formula (11)][60].

NOTE Experimental standard deviations can vary considerably by chance. It can be useful to calculate the standard deviation expected based on Poisson statistics to confirm that the experimental standard deviation does not give an unrealistically small indication of the uncertainty.

Consideration shall also be given to the following factors specifically affecting dPCR-based measurement procedures:

a) **Partition volume uncertainty**. This element is required for dPCR quantification of DNA copy number concentration (see 4.2.3). For ratio based dPCR measurements (see 4.2.5), partition volume uncertainty is not required, however in particular cases of high lambda values, the heterogeneity of partition volume within a reaction can contribute to the uncertainty in the result of a ratio-based measurement[59][61]. Published studies can be used to estimate partition volume uncertainty[62][63][64][65]. The partition volume value applied should be appropriate to the type of dPCR reagents (mastermix) employed[64].

b) **Classification of positive and negative partitions.** Variation in fluorescent threshold setting influences the quantification of both copy number concentration and ratios by dPCR[58][66]. Therefore, the contribution of this source of uncertainty should be estimated from data where the threshold setting has been varied for a single data set; for example, analysis by different operators or comparison of alternative software in use.

11 Reporting

The data report shall contain sufficient detail to allow independent assessment of the nucleic acid quantification results.

Reporting elements should include, but are not limited to:

— sample — ID, source descriptors;

— reagents — name, source, lot number;

— oligonucleotide synthesis — manufacturer, purity;

— sample preparation procedures and conditions;

— make and model of instrument used and relevant settings — including PCR thermal cycling conditions and total reaction volume;

— qualification, validation, and verification plans;

— measurement results with appropriate units and uncertainty;

— data analysis procedure;

— unexpected observations.

Specific methodological and performance parameters which should be detailed for qPCR and dPCR, together with an indication of priority (essential/desirable), in accordance with the MIQE[1] and dMIQE[67] guidelines are described in Annex E.

Annex A
(informative)

Spectrophotometry

A.1 General

Spectrophotometry is a commonly used method for total nucleic acid quantification (see 5.2.2) and assessment of nucleic acid purity (see 5.4). This annex provides information on absorbance coefficients for total nucleic acid concentration measurements, criteria for interpreting chemical purity and examples of results from typical UV-spectrophotometry analysis.

A.2 Relationship between absorbance and total nucleic acid concentration

The concentration of nucleic acid is determined using the Beer-Lambert law, which predicts a linear correlation between absorbance at a wavelength of 260 nm and nucleic acid concentration, with optical density coefficient dependent on the nucleic acid species quantified (double-stranded (ds)DNA, single-stranded (ss)DNA, single stranded (ss)RNA, double stranded (ds)RNA).

An absorbance reading at 260 nm of 1,0 is equivalent to ~40 µg/ml of pure (ss)RNA, ~46 µg/ml of pure (ds)RNA and ~50 µg/ml of pure (ds) DNA. More rigorous nucleic acid quantification can be performed under denaturing conditions with ssDNA showing an absorbance coefficient of 37 µg/ml.

A.3 Absorbance ratios and nucleic acid purity

For a high-quality DNA sample, the ratio of the absorbance readings at 260 nm and 280 nm (260/280 ratio) for pure DNA should be close to 1,8. If the ratio is appreciably lower it can indicate the presence of protein, phenol or other contaminants. If the ratio is close to 2, this can indicate RNA contamination.

For a high-quality RNA sample, the 260/280 ratio should be close to 2. It has been observed that a ratio of the absorbance readings at 260 nm and 280 nm of 1,8 suggests there is about 70-80 % of protein in the samples. As there are many proteins that inhibit both PCR and reverse transcription, the laboratory should define its thresholds to reject or accept a sample.

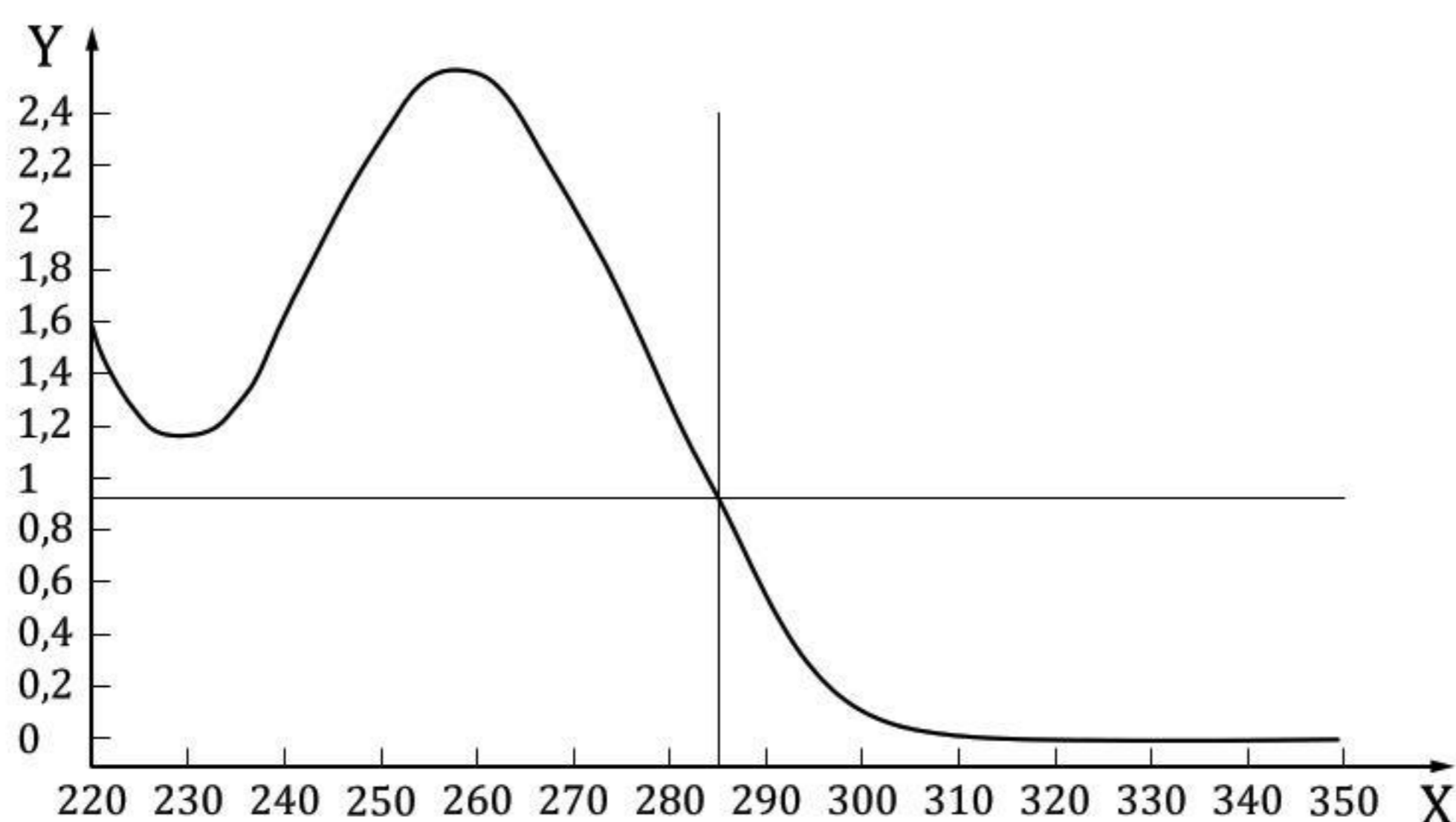
It should be noted that the 260/280 ratio can vary with pH and ionic strength[68].

The ratio of the absorbance reading at 260 nm to the absorbance reading at 230 nm (260/230 ratio) should ideally be in the range of 2,0 to 2,2. If the ratio is appreciably lower than expected, it can indicate the presence of contaminants which absorb at 230 nm.

The baseline absorbance reading should be subtracted from all measurements (even in case of a ratio).

Further guidance is provided in ISO 21571.

Figures A.1 and A.2 show examples of DNA samples of different quality. The good quality DNA sample in Figure A.1 demonstrates a 260/280 ratio of between 1,8 and 2,0 and a 260/230 ratio of 2,20. The poor quality DNA sample in Figure A.2 does not show a defined peak at 260 nm together with absorbance at 280 nm and a large peak at 230 nm resulting in a very low 260/230 ratio (0,33) and a 260/280 ratio < 1,8.

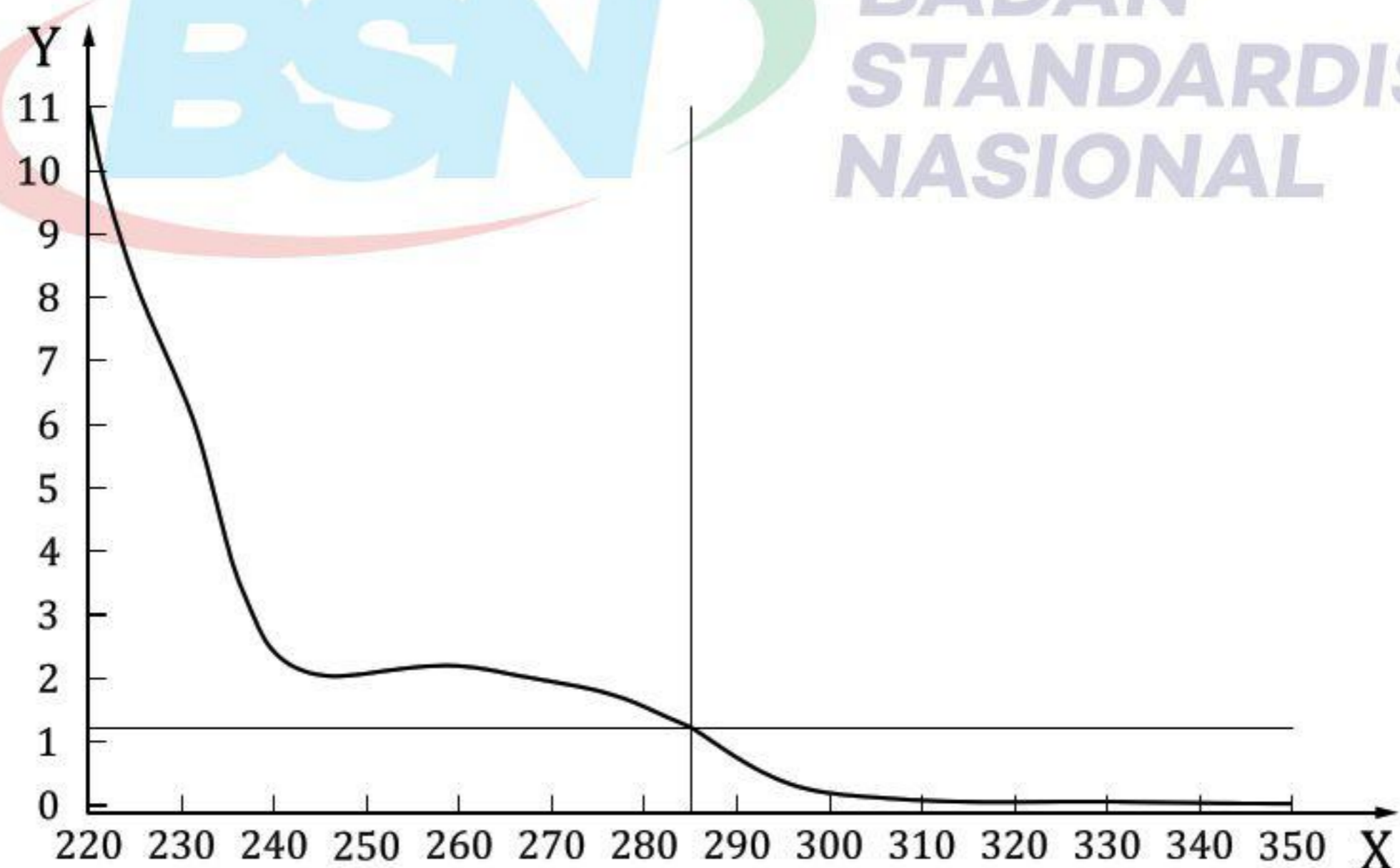
Key

X wavelength, in nm

Y absorbance

Figure A.1 — Example of spectrophotometric readout for a high-quality DNA sample

Absorbance in the range 220 nm to 350 nm is shown alongside output for DNA concentration (ng/μl) and absorbance ratios.

Y
11
10
9
8
7
6
5
4
3
2
1
0
220 230 240 250 260 270 280 290 300 310 320 330 340 350 X

Key

X wavelength, in nm

Y absorbance

Figure A.2 — Example of spectrophotometric readout for a poor-quality DNA sample

Absorbance in the range 220 nm to 350 nm is shown alongside output for DNA concentration (ng/μl) and absorbance ratios.

Annex B
(informative)

Nucleic acid integrity

B.1 General

Investigation of nucleic acid integrity enables the assessment of factors which can influence the performance of a qPCR- or dPCR-based measurement procedure, such as the presence of nucleic acid degradation (short fragments) or high molecular weight DNA (see 5.3). Methods for evaluation of the fragment size distribution of DNA or RNA in a sample are listed in B.2 and B.3 respectively. Examples of methods for analysis of nucleic acid integrity analysis applied to a range of clinical samples are provided in Reference [20].

B.2 Techniques for evaluation of DNA integrity

B.2.1 Electrophoresis

For cellular gDNA, a high molecular weight band is normally present at the top of the gel. Smears or comet tails are indicative of sample degradation and fragmentation. Conversely, for cell-free DNA, a main peak fragment size of approximately 130 - 170 base pairs (bp) is expected[69]. For some instruments, specific algorithms provide a quantitative index of the DNA integrity, based on the electrophoretic run features.

B.2.2 Long range PCR

Long range PCR can be used to amplify sequences up to 15 kb, using specific types of polymerases. It gives a qualitative estimation of DNA integrity[70].

B.2.3 PCR assay with differential size amplicons

The same genomic region is targeted with multiple PCR primer sets of varying amplicon sizes and provides an index or ratio of the amplifiable molecules at different fragment sizes[71][72][73][74].

B.3 Techniques for evaluation of RNA integrity

B.3.1 Electrophoresis

Electrophoretic profiles present distinct bands for different molecule groups ribosomal RNA (rRNA) of different sizes, small RNA etc.) and show a shift in fragment size associated with degradation. Specific algorithms provide a quantitative index of the RNA integrity, based on the electrophoretic run features[75].

Algorithms that are based mainly on the electrophoretic run features of rRNA are fit for purpose for the assessment of the integrity of RNA molecules of high quality, where rRNA peaks are discernible. For prokaryotic RNA, the rRNA ratio of 23S and 16S ribosomal subunits is used for integrity estimation. For eukaryotic RNA, the rRNA ratio of 28S rRNA and 18S ribosomal subunits is used. However, if RNA quality is severely compromised (e.g. after tissue formalin fixation), these algorithms are not fit for purpose for the assessment of the quality of mRNA molecules.

B.3.2 RT-qPCR for 5′/3′ mRNA ratio

This integrity assay relies on measuring the ratio of the quantities of the target located at the 5′ and target located at the 3′ of the same transcript using oligo dT-based RT priming. This provides a relative measure of mRNA integrity. If the RNA is intact an equal concentration is expected using each assay (ratio = 1), whereas a higher copy number of the 3′ assay relative to the 5′ is expected if the RNA is degraded[76].

The efficacy of a 3′ based RT priming strategy should be validated using typical test samples as it can be the case that it is not informative for highly fragmented RNA.

B.3.3 RT-qPCR assay of repetitive elements

The concentration of expressed repeat sequences (e.g. AluJ) can be measured as an indication of mRNA amplifiability[76]. A control sample of high integrity should be included to give a robust indication of sample degradation.

B.3.4 RT-qPCR assays with different size amplicons

As for DNA integrity, this approach can be applied to RNA integrity by measuring the same transcript with multiple size amplicons[77].

Annex C
(informative)

PCR efficiency

C.1 General

The PCR efficiency of a qPCR assay is normally tested by constructing a calibration curve or dilution series (see 6.2.3). This annex provides information on experimental design and calculation of PCR efficiency.

C.2 Experimental design

For evaluation of qPCR efficiency, the calibration materials or sample for preparation of a dilution series ('standard solutions') forming the standard curve should consist of purified template DNA. These could be synthetic oligonucleotides, purified plasmid, purified gDNA or purified PCR product. For evaluation of RT-qPCR efficiency, the standard solutions should consist of purified template RNA: for example, in vitro transcribed RNA or purified cellular RNA. The standard solutions can be produced from a high concentrated stock that is diluted serially.

A carrier nucleic acid (with no cross-reactivity to the assay) is recommended to keep the total nucleic acid content of a reaction equivalent through the dilution series, particularly for molecules with low molecular weight such as oligonucleotides or plasmid DNA, which can adhere to tubes. The standard curve shall cover as wide concentration range as possible and be applicable to the intended use of the measurement procedure.

A precise estimate of PCR efficiency is characterized by a narrow confidence interval. This is obtained using large number of standards. A minimum of 24 measurements, distributed, for example, as four replicates at six different concentrations, is recommended[78].

C.3 Calculations

The following quality measures and test parameters are suggested:

a) Replicate data are tested for the presence of outliers. Outliers can be identified, for example, with Grubb's test of the samples relative to the residual. Using 95 % confidence is recommended.

   1) If a single outlier is detected, data and analysis shall be reviewed to identify the cause for the outlier. If the cause is identified and can be excluded, the outlier can be removed from the analysis and the remaining data can be used to construct the standard curve;

   2) If more than one outlier is detected for a sample, the data corresponding to this sample should not be used to construct the standard curve.

b) The qPCR data from the 'standard solutions' are fitted to Formula (C.1) by linear regression:

$$y = a + bx \tag{C.1}$$

where

y is the C_q value;

x is the logarithm to the base 10 of the concentration (($\log_{10}(c_i)$) or relative concentration (in the case of a dilution series) of the standard solution;

a is the intercept;

b is the gradient.

The correlation coefficient of the linear regression shall be evaluated (see 6.2.3).

Additional testing for linearity can be performed, for example, by evaluating with run tests or comparing the linear fit to fits to second and third order polynomials [Formulae (C.2) and (C.3)].

$$y = a + bx + cx^2 \tag{C.2}$$

$$y = a + bx + cx^2 + dx^3 \tag{C.3}$$

where

c is the coefficient of the second order term;

d is the coefficient of the third order term.

The importance of the fitted parameters c and d is compared to the slope b. Significant weighting of c and d suggest deviation from linearity at either low or/and high concentration.

If data show deviation from linearity the extreme data point shall be removed and the remaining data, now covering a narrower range, shall be inspected for linearity.

For qPCR data deviation from linearity is frequently seen at high concentration, particularly on certain instruments. This deviation can be due to incorrect subtraction of the baseline by the instrument software due to early increase of fluorescence of the most concentrated sample. Deviation from linearity can have profound effect on the estimation of PCR efficiency, as the deviant samples are at extreme concentrations and, because of the heavier effect of the linear regression, have profound influence on the fit, resulting in estimates of PCR efficiency that are significantly above 100 %, which is theoretically impossible. Adjustments to the baseline setting approach should be made if possible, or the measurement procedure validated over a narrow range of concentrations (see 6.3.3).

c) Mean PCR efficiency ($\bar{E}$) is estimated from multiple experiments with mean gradient ($\bar{b}$) [Formula (C.4)]:

$$\bar{E} = 10^{-1/\bar{b}} - 1 \tag{C.4}$$

with standard error (SE) [Formula (C.5)]:

$$SE_{\bar{E}} = SE_{\bar{b}} \times \frac{\left(1+\bar{E}\right) ln10}{\bar{b}^2} \quad \text{(C.5)}$$

and confidence interval (CI) [Formula (C.6)]:

$$CI = \bar{E} \pm t_{95\ \%, n-2} \times SE_{\bar{E}} \quad \text{(C.6)}$$

where

$t_{95\ \%, n-2}$ is the expansion factor at 95 % confidence level calculated from the inverse of the t-distribution;

n is the number of experiments performed to determine PCR efficiency.

Annex D
(informative)

Measurement uncertainty

D.1 General

Both processing of a biological sample (sampling from source material, storage, nucleic acid extraction) and nucleic acid measurement influence the uncertainty in the quantity value obtained by a qPCR or dPCR-based measurement procedure. Effects can be systematic; associated with a positive or negative bias (for example, nucleic acid extraction or RT efficiency of < 100 %) or random (related to precision of the techniques applied). A nested experimental design with replication for the different stages in the complete process informs precision and predicts random effects on measurement uncertainty in the result[79].

An overview of sources of uncertainty which can influence qPCR and dPCR measurements is provided in D.2 and D.3 to aid qPCR or dPCR method designers in considering factors which can be relevant in developing an appropriate measurement uncertainty budget.

D.2 Sources of uncertainty for whole process (qPCR)

Possible sources of uncertainty in a whole process from biological sample to nucleic acid quantification are illustrated in Figure D.1 for a qPCR-based measurement procedure. For RT-qPCR, unique sources of uncertainty include DNase treatment and RT. For qPCR methods employing a calibration curve (see 4.2.2), sources of uncertainty in the value assigned to the calibration material are relevant.

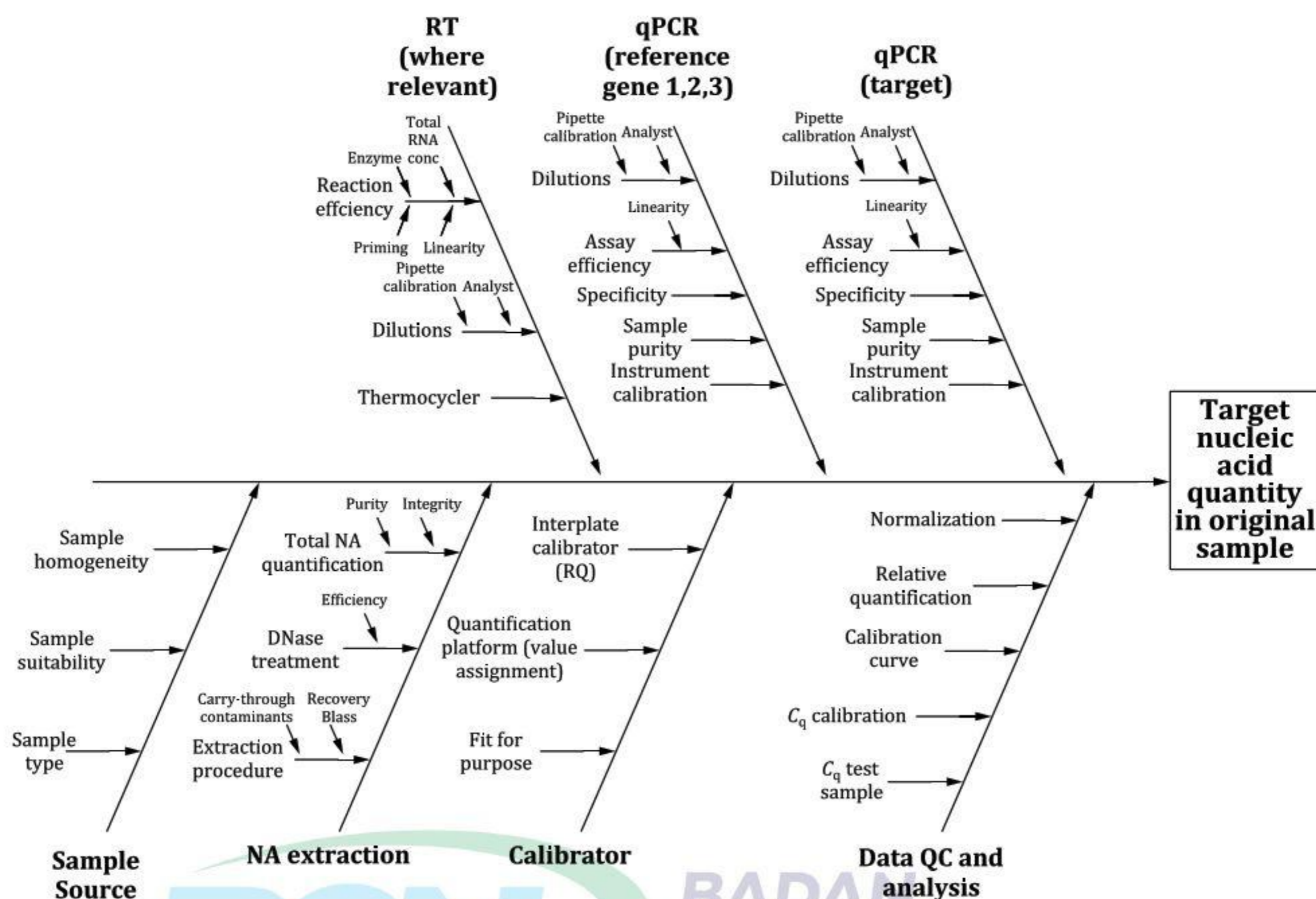



Figure D.1 — Cause and effect diagram — Whole process uncertainty contributions for a qPCR-based measurement procedure

The central arrow represents the experimental process from sample to the quantification method. Branches feeding into experimental progression characterize sources of variability that contribute to uncertainty at various stages of the process. Adapted from Reference [80].

D.3 Sources of uncertainty for dPCR

Sources of uncertainty influencing dPCR are illustrated in Figure D.2 for a droplet dPCR-based measurement procedure applied to the quantification of copy number concentration (see 4.2.3).

Key

$C_{primers/probe}$	concentration primers and probe
Df_{sample}	dilution factor of sample before addition to PCR mix
Df_{PCR}	dilution factor of sample in the PCR mix
M_{dil}	mass of diluent
$M_{dil+sample}$	mass of diluent and sample
M_{premix}	mass of pre sample mix
M_{mix}	mass of the PCR mix with sample
V_d	volume of the droplets

NOTE Reproduced with permission from Reference [58].

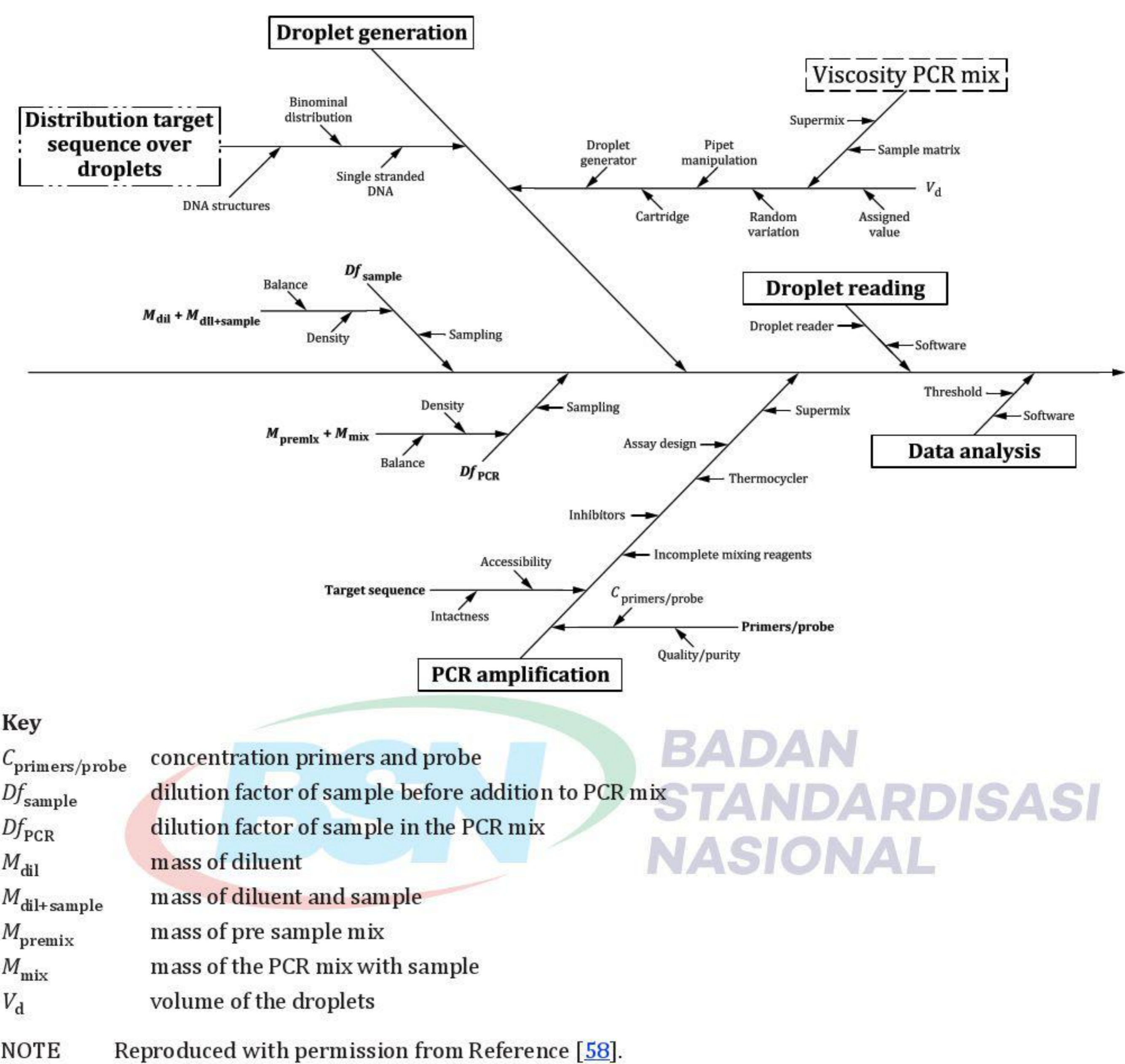



Figure D.2 — Cause and effect diagram — Uncertainty contributions for a dPCR-based measurement procedure

Annex E
(informative)

MIQE and dMIQE checklists

E.1 General

The information requirements for qPCR or dPCR method development in accordance with this document share many of the requirements for publication of experimental data from qPCR and dPCR experiments outlined in the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) and Minimum Information for Publication of Quantitative Digital PCR Experiments (dMIQE) guidelines[1][67]. The MIQE and dMIQE checklists are reproduced here to inform qPCR and dPCR method developers of the aspects of the whole process (including sampling, nucleic acid extraction, measurement) which can be described.

E.2 MIQE checklist

Table E.1 details the methodological and performance parameters detailed in the checklist for publication of qPCR-based experimental results published in the MIQE Guidelines[1].

Table E.1 — MIQE checklist [reproduced with permission of the American Association for Clinical Chemistry (AACC)]

Item to check	Importance
EXPERIMENTAL DESIGN	
Definition of experimental and control groups	E
Number within each group	E
Assay carried out by core lab or investigator's lab?	D
Acknowledgement of authors' contributions	D
SAMPLE	
Description	E
Volume/mass of sample processed	D
Microdissection or macrodissection	E
Processing procedure	E
If frozen - how and how quickly?	E
If fixed - with what, how quickly?	E
Sample storage conditions and duration [especially for formaline fixed and paraffin embedded (FFPE) samples]	E
NUCLEIC ACID EXTRACTION	
Procedure and/or instrumentation	E
Name of kit and details of any modifications	E
Source of additional reagents used	D
Details of DNase or RNAse treatment	E
Contamination assessment (DNA or RNA)	E

Key

E essential

D desirable

Table E.1 *(continued)*

Item to check	Importance
Nucleic acid quantification	E
Instrument and method	E
Purity (A260/A280)	D
Yield	D
RNA integrity method/instrument	E
RIN/RQI or C_q of 3' and 5' transcripts	E
Electrophoresis traces	D
Inhibition testing (C_q dilutions, spike or other)	E
REVERSE TRANSCRIPTION	
Complete reaction conditions	E
Amount of RNA and reaction volume	E
Priming oligonucleotide (if using GSP) and concentration	E
Reverse transcriptase and concentration	E
Temperature and time	E
Manufacturer of reagents and catalogue numbers	D
C_qs with and without RT	D
Storage conditions of cDNA	D
qPCR TARGET INFORMATION	
Sequence accession number	E
Location of amplicon	D
Amplicon length	E
In silico specificity screen (BLAST, etc.)	E
Pseudogenes, retropseudogenes or other homologues?	D
Sequence alignment	D
Secondary structure analysis of amplicon	D
Location of each primer by exon or intron (if applicable)	E
What splice variants are targeted?	E
qPCR OLIGONUCLEOTIDES	
Primer sequences	E
RTPrimerDB Identification Number	D
Probe sequences	D
Location and identity of any modifications	E
Manufacturer of oligonucleotides	D
Purification method	D
qPCR PROTOCOL	
Complete reaction conditions	E
Reaction volume and amount of cDNA/DNA	E
Primer, (probe), Mg++ and dNTP concentrations	E
Polymerase identity and concentration	E
Buffer/kit identity and manufacturer	E
Exact chemical constitution of the buffer	D

Key

E essential

D desirable

Table E.1 *(continued)*

Item to check	Importance
Additives (SYBR Green I, DMSO, etc.)	E
Manufacturer of plates/tubes and catalogue number	D
Complete thermocycling parameters	E
Reaction setup (manual/robotic)	D
Manufacturer of qPCR instrument	E
qPCR VALIDATION	
Evidence of optimization (from gradients)	D
Specificity (gel, sequence, melt, or digest)	E
For SYBR Green I, *C*q of the NTC	E
Standard curves with slope and y-intercept	E
PCR efficiency calculated from slope	E
Confidence interval for PCR efficiency or standard error	D
R^2 of standard curve	E
Linear dynamic range	E
C_q variation at lower limit	E
Confidence intervals throughout range	D
Evidence for limit of detection	E
If multiplex, efficiency and LOD of each assay	E
DATA ANALYSIS	
qPCR analysis program (source, version)	E
C_q method determination	E
Outlier identification and disposition	E
Results of NTCs	E
Justification of number and choice of reference genes	E
Description of normalization method	E
Number and concordance of biological replicates	D
Number and stage (RT or qPCR) of technical replicates	E
Repeatability (intra-assay variation)	E
Reproducibility (inter-assay variation, % CV)	D
Power analysis	D
Statistical methods for result significance	E
Software (source, version)	E
C_q or raw data submission using Real-time PCR Data Markup Language (RDML)	D

Key

E essential

D desirable

E.3 dMIQE checklist

Table E.2 details the methodological and performance parameters detailed in the checklist for publication of dPCR-based experimental results published in the dMIQE Guidelines.

Table E.2 — dMIQE checklist [reproduced with permission of the American Association for Clinical Chemistry (AACC)]

Item to check	Importance
EXPERIMENTAL DESIGN	
Definition of experimental and control groups	E
Number within each group	E
Assay carried out by core lab or investigator's lab?	D
Power analysis	D
SAMPLE	
Description	E
Volume or mass of sample processed	E
Microdissection or macrodissection	E
Processing procedure	E
If frozen - how and how quickly?	E
If fixed - with what, how quickly?	E
Sample storage conditions and duration (especially for FFPE samples)	E
NUCLEIC ACID EXTRACTION	
Quantification - instrument/method	E
Storage conditions: temperature, concentration, duration, buffer	E
DNA or RNA quantification	E
Quality/integrity-instrument/method; e.g. RIN/RQI and trace or 3’:5’	E
Template structural information	E
Template modification (digestion, sonication, pre-amplification etc.)	E
Template treatment (initial heating or chemical denaturation)	E
Inhibition dilution or spike	E
DNA contamination assessment of RNA sample	E
Details of DNase treatment where performed	E
Manufacturer of reagents used and catalogue number	D
Storage of nucleic acid: temperature, concentration, duration, buffer	E
REVERSE TRANSCRIPTION (If necessary)	
cDNA priming method + concentration	E
One or two step protocol	E
Amount of RNA used per reaction	E
Detailed reaction components and conditions	E
RT efficiency	D
Estimated copies measured with and without addition of RT	D
Manufacturer of reagents used and catalogue number	D
Reaction volume (for two step reverse transcription reaction)	D
Storage of cDNA: temperature, concentration, duration, buffer	D
dPCR TARGET INFORMATION	
Sequence accession number	E
Location of amplicon	D
Amplicon length	E

Key

E essential

D desirable

Table E.2 *(continued)*

Item to check	Importance
In silico specificity screen (BLAST, etc.)	E
Pseudogenes, retropseudogenes or other homologues?	D
Sequence alignment	D
Secondary structure analysis of amplicon and GC content	D
Location of each primer by exon or intron (if applicable)	E
Where appropriate, which splice variants are targeted?	E
dPCR OLIGONUCLEOTIDES	
Primer sequences and/or amplicon context sequence	E
RTPrimerDB Identification Number	D
Probe sequences	D
Location and identity of any modifications	E
Manufacturer of oligonucleotides	D
Purification method	D
dPCR PROTOCOL	
Complete reaction conditions	E
Reaction volume and amount of RNA/cDNA/DNA	E
Primer, (probe), Mg++ and dNTP concentrations	E
Polymerase identity and concentration	E
Buffer/kit Catalogue No and manufacturer	E
Exact chemical constitution of the buffer	D
Additives (SYBR Green I, DMSO, etc.)	E
Plates/tubes Catalogue No and manufacturer	D
Complete thermal cycling parameters	E
Reaction setup	D
Gravimetric or volumetric dilutions (manual/robotic)	D
Total PCR reaction volume prepared	D
Partition number	E
Individual partition volume	E
Total volume of the partitions measured (effective reaction size)	E
Partition volume variance/standard deviation	D
Comprehensive details and appropriate use of controls	E
Manufacturer of dPCR instrument	E
dPCR VALIDATION	
Optimization data for the assay	D
Specificity (when measuring rare mutations, pathogen sequences etc.)	E
Limit of detection of calibration control	D
If multiplexing, comparison with singleplex assays	E
DATA ANALYSIS	
Average copies per partition (λ or equivalent)	E
dPCR analysis program (source, version)	E
Outlier identification and disposition	E

Key

E essential

D desirable

Table E.2 *(continued)*

Item to check	Importance
Results of NTCs	E
Examples of positive(s) and negative experimental results as supplemental data	E
Where appropriate, justification of number and choice of reference genes	E
Where appropriate, description of normalization method	E
Number and concordance of biological replicates	D
Number and stage (RT or qPCR) of technical replicates	E
Repeatability (intra-assay variation)	E
Reproducibility (inter-assay/user/lab etc. variation)	D
Experimental variance or confidence interval	E
Statistical methods used for analysis	E
Data submission using RDML	D

Key

E essential

D desirable

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